REMARKS

Claims 58-193 are pending in the subject application. Claims 58, 77-79, 86, 88-90, 101, 105, 126, 162, 172, 183, 186-191 and 193 have been amended for clarification purposes. Claims 194-198 have been added. Claim 99 has been canceled. Support for the amendment to claims 58, 77-79, 86, 88-90, 101, 105, 126, 162, 172, 183, 186-191 and 193 and for added claims 194-198 is found throughout the Specification, as filed, and no new matter is presented by the amendment.

Favorable reconsideration in light of the amendments and remarks which follow s respectfully requested.

1. 35 U.S.C. §112 Rejections

35 U.S.C. §112, first paragraph

Claims 58-193 have been rejected under 35 U.S.C. §112, first paragraph, because

> the specification, while being enabling for specific combinations of substances (first substance, second substance and third substance), specific liquid medium, does not reasonably provide enablement for claims as claimed with confusing terminology.

In particular, the Office asserts that:

> the three substances are not clearly defined and differentiated in the specification. There is a considerable overlap between the classes and it is unclear how one class forms more extended surfaces than the other (there is no very specific definition of extended surfaces either; it is well known in the art that the amphipathic substances form a bilayer structure). The first and second class substances include phospholipids and it is unclear how the phospholipids are more soluble and less soluble in an aqueous suspending medium.

Applicants claim, in claim 58, three "classes" of amphipathic substances:

(1) first surface-building amphipathic substance selected from lipids, lipid-like materials and combinations thereof

(2) second surface-destabilising amphipathic substance selected from surface-active substances, surfactants and combinations thereof
(3) third amphipathic substance selected from chain molecules and macromolecules

As set out in claim 58, the first substance and the second substance form extended surfaces in contact with the medium. The solubility of the second substance in the liquid medium is greater than the solubility of the first substance in the liquid medium. Further, the molecules of the third substance associate with the extended surfaces formed by the first substance and the second substance. The presence the second substance in the combination increases the ability of the extended surfaces formed by the first substance and the second substance to associate molecules of the third substance. Further, the at least one first, at least one second and at least one third substance are different from each other.

Applicants respectfully submit that while it is true that the first substance and the second substance cab both be phospholipids, this does not necessarily lead to an overlap as asserted by the Office. The class of phospholipids comprises a multitude of completely different compounds having only one common structural feature: they are all phosphoric acid esters. The properties of each individual phospholipid, such as solubility, capability to build surfaces, etc., therefore is a function of its individual characteristics and number of substituents attached to the phosphoric ester unit. The more apolar the substituents are, the less soluble the phospholipid will be. The same applies to apolar substituents. Thus, in a group of compounds, all of which are phospholipids, some will be well-soluble in aqueous media, while others will be nearly insoluble. This is well-known to a person skilled in the art. It is also common knowledge to one skilled in the art what a molecule must look like (short or extended substituents, polar or apolar groups) in order for it to be more or less soluble in aqueous media.

In the present invention, it is specified that the first substance can be selected from, e.g., glycerophospholipids, namely phosphatidylcholines, phosphatidylethanolamines, phosphatidylglycerols, phosphatidylinositols, phosphatidic acids, and phosphatidylserines. All of these substances have extended apolar substituents in position 1 and 2, and at the phosphoryl group in position 3. Also mentioned are sphingolipids like sphingophospholipids and sphingoglycolipids. These substances also have extended apolar groups. The provision that all of these lipids are capable of forming bilayers is set forth by Applicants (See p. 18, lines 1 to 11). These types of phospholipids are known to be poorly water-soluble due to their apolar portions, and are known to be capable of building membrane-like structures by aggregating upon solution or dispersion in a polar liquid medium.

In contrast, the only phospholipids mentioned for use as the second substance are lysophospholipids, i.e. phospholipids, from which at least one of the extended acyl-(fatty acid)groups is eliminated. Thus, lysophospholipids are markedly more polar than the phospholipids specified for the first substance. As a result the second substances have a higher solubility in polar media and surfactant like properties, i.e. structure destabilising properties, or the capability of forming only relatively small surfaces like, e.g., micelles. This is well-known to one of skill in the art.

Applicants respectfully submit that the different aggregation and solubility properties of both of these phospholipid groups is also described in several publications, which clearly show that phospholipids with a single "fluid" chain ("lysophospholipids" of synthetic (Maurer et al., 1994) or quasi-biological (Jain et al., 1985) origin) form spherical micellar aggregates with rather small surface areas (according to Maurer et al., 1994: , $r \sim 3.8$ nm, i.e. $S \sim 181$ nm^2). In contrast, typical double (fatty acid) chain phospholipids aggregate into at least 10 times larger vesicles (Hauser, *Phospholipids Handbook:* $r > 12$ nm; $S > 1800$ nm^2) and most often form much more extended bilayers.

The significant difference in water solubility of various single and double chain phospholipids, as expressed in terms of critical micellisation or aggregation concentration (CMC or CAC, respectively) is further evident from *Phospholipids Handbook*, and from the article by D. Marsh (Marsh & King, 1986).

Thus, Applicants respectfully submit that there is no overlapping between the phospholipids mentioned in class 1, and the lysophospholipids mentioned in class 2. The characteristic properties of each of these substance groups are well-known in the art, and, therefore allow an unambiguous classification of these substances into the class of bilayer-forming substances and more soluble, surface-destabilising substances or substances, which only form small surfaces.

The Office further asserts that:

> It is unclear as to how surfactants such as Tritons (second substance) can form extended surface by themselves when they are water-soluble and form solutions.

Applicants respectfully disagree. Applicants submit that claim 58 claims only that the second substance is more soluble than the first substance is in the liquid medium. Thus, this is merely a relative feature and it does not mean that the second substance is dissolved in the medium. Rather, it only states that the second substance is more soluble than the first substance. As previously explained, solubility and the capability of forming surfaces are not mutually exclusive. This is, for example, a question of the concentration of the dissolved substance, as well as of inter-molecular interactions like van-der-Waals forces, etc. Thus, a substance may be soluble in a specific medium, and, under certain circumstances, also be capable of aggregating and forming surfaces, at the same time.

A typical example of this phenomenon are micelles, which are generally defined as aggregates formed by the association of dissolved molecules. Most commonly micelles are aggregates being formed by surfactant molecules in aqueous solution above a specific temperature (Krafft point) and a characteristic concentration (cmc).

These micelles have diameters of few to about 100 nm. (CD Römpp Chemie Lexikon, 9th ed., Georg Thieme Verlag, Stuttgart 1995, "Mizellen").

In contrast, surface-building substances, like the phospholipids mentioned with relation to the first substance, are capable of forming more extended surfaces than those of the second substance, e.g. in the form of liposomes or other vesicles, which are defined as spherical structures with diameters of 25 nm to up to 1 mm, consisting of one or more concentric lipid bilayers surrounding an aqueous interior (CD Römpp Chemie Lexikon, 9th ed., Georg Thieme Verlag, Stuttgart 1995, "Liposomen").

This also explains what is meant by substances forming more extended surfaces (less soluble 1st substance) and substances forming less extended surfaces (more soluble 2nd substance).

In view of the above, and since all of the above definitions and explanations are common knowledge, which can be found in most of the standard chemical or pharmaceutical encyclopedia, Applicants respectfully submit that the present disclosure along with the common definitions of said substances unambiguously enables a person skilled in the art to determine the substances needed for carrying out the present invention.

The Office further asserts that:

> With regard to chain molecules (third substance), one of ordinary skill in the art would not be able to determine which of the compounds come under this category since even sterols which do not have chain moieties are recited in the specification as belonging to this class.

And, further, that

> According to the claims and the specification, the second and third substance can be the same. If so, it is unclear as to how one can form the extended surface and the other does not, but attaches itself on the second substance.

Applicants respectfully submit that, upon close review of the specification, there is no description of the third substance being a sterol. Applicants respectfully request reconsideration and withdrawal of this rejection.

Applicants further note that claim 99, which was directed to an embodiment wherein the second and third substances may be the same, has been deleted, thus the rejection regarding claims to an embodiment wherein the second and third substance are the same is moot.

The Office further asserts that:

> The specification also does not provide a clear definition of 'edge active substance' and how it is distinguished from surfactant. In fact, the examiner points out that even phospholipids are considered as surfactants. Finally, it should be pointed out that the specification describes one methodology and it is unclear as to how by this method alone one can form different extended surfaces.

Applicants respectfully submit that the term "edge active" has been replaced with its corresponding term "surface active". The terms "edge active" and "surface active" are broader than the term "surfactant". Surfactants only cover a specific group of chemical compounds like anionic or cationic surfactants, etc. However, surfactants do not, or example, include low alcohols, which do come under the definition of "edge-active" or "surface-active" substances. The Office's example of phospholipids demonstrates this problem very clearly. Phospholipids commonly are not classified as surfactants, but they may have edge-active (surface-active) properties, if they are correspondingly substituted.

The Examiner also noted that there is no specific definition of extended surfaces, and that it would be well-known in the art that the amphipathic substances form bilayer structures.

Regarding the Office's assertion that there is no specific definition of extended surfaces, Applicants respectfully submit that it appears that the Office objects to the term "extended" and does not have any objection to the term "surface" (edge active vs. surface active). Applicants further submit that in the present specification, it is explicitly mentioned which surfaces are "extended" surfaces:

> A surface, in the context of this document, is deemed to be extended, if it allows propagation and/or evolution of co-operative surface excitations in two dimensions. The surface of a vesicle, for example, fulfils this criterion by supporting surface undulations or fluctuations; depending on membrane flexibility, average vesicle diameters between 20 nm and several hundred nanometres are needed for this. (Mixed) lipid micelles, which do not reach this dimension at least in one direction, do not fulfil the requirement; if so, their surface is not considered to be extended in the sense of this invention" (See page 14, lines 14-21).

Applicants respectfully submit that the term "extended surfaces" is, in fact, clearly defined in the specification. Further, in addition to the above definition, quantitative definitions are given in the specification as the average radius of the extended surfaces - e.g. between 15 nm and 5000 nm (see page 16, lines 16-26). Thus, the surface can be calculated by the formula for surfaces of spheres, $S = 4\pi r^2$, giving a surface of about 2827 nm^2 to 314 μm^2 for an average radius of from 15 to 5000 nm.

Thus, the extended surfaces are clearly qualitatively defined by their constituents (which the Office has acknowledged by stating that it is well known in the art that amphipathic substances form bilayer structures), and quantitatively by their radius.

Regarding the Office's assertion that it is unclear how by one methodology alone one can form different extended surfaces, Applicants respectfully submit that this is clearly set forth in the Examples of the present specification, all of which reflect the claimed preparation method:

(1.) selecting two amphiphilic substances being basically capable of forming surfaces in the selected medium and differing in their solubility in this medium.

(2.) combining said substances in a medium, such that extended surfaces are formed.

More detailed steps for obtaining this combination and for the induction of the formation of extended surfaces as mentioned in the subclaims are described in the Examples (See, e.g. page 32, line 8 - page 33, line 24), and the further Examples. These examples, all of which employ the same preparation method, indeed provide different surfaces, as they are using different components, different concentrations, different ratios, different agents. Thus, it is clear that, although the same method is used, different surfaces are obtained.

Thus, Applicants respectfully submit that it is not unclear, how different surfaces may be formed by the same methodology. Other wise, it would have to be surprising, as well, that, for example, common chemical reactions like Grignard reactions provide a variety of different products when different components are used.

35 U.S.C. §112, second paragraph

Claims 58-193 have been rejected under 35 U.S.C. §112, second paragraph, as being indefinite. In particular, the Office asserts that:

> Applicant cancels the originally presented claims and presents new claims with the same issues which existed before without addressing the issues. For example, as already indicated, if a substance is soluble in a liquid (regardless of the degree of solubility) how can it form an extended surface? What is meant by an extended surface? If all three are amphipathic substances, why would only two form extended surfaces and the third one not? What is the solubility of the third amphipathic substance? The examiner had already suggested naming these substances and the third substances. 'less extended', 'more extended' are indefinite since they are relative terms.

Applicants respectfully traverse and submit that the above-discussion addresses many of these issues raised by the Office.

Regarding how a substance can form extended surfaces if it is soluble in a liquid (regardless of the degree of solubility) and what is meant by an extended

surface, Applicants respectfully submit that both of these questions were answered by the above explanations concerning the formation of micelles and liposomes, etc., and the determination of extended surfaces, respectively.

Regarding the Office's question of what an "extended surface" is, Applicants have answered this question above. In particular, Applicants explicitly describe which surfaces are "extended" in the Specification (see page 14, lines 14-21; page 16, lines 16-26), both qualitatively in terms of their constituents and quantitatively in terms of their radius.

Regarding why only two of the three substances form extended surfaces, although all of them are amphipathic, Applicants respectfully submit that the third substance (i.e. extended chain or macromolecules capable of adsorbing onto extended surfaces comprising at least two different kinds of amphiphiles), do not form extended aggregates on their own due to the following well-known basic rules for aggregation: If chain molecule or macromolecule adsorption is to be superficial and reversible, the hydrophobic portions of such molecule, which contribute to the binding energy, must be relatively small and distributed along the chain. If an exposed hydrophobic portion of a chain molecule reaches a segmental length comparable to bilayer thickness, which is typically 3-5 nm, such a segment will have a tendency to become inserted into rather than adsorbed onto lipid bilayers with extended surface. Alternatively, a chain molecule or macromolecule with extended hydrophobic segments may also form homo-aggregates, in which the hydrophobic segments are not exposed to the aqueous medium and, thus, are no longer available for adsorption to the surface of mixed amphipath aggregates.

Therefore, the third substances mentioned in the present invention only comprises chain molecules or macromolecules having relatively small hydrophobic portions, which are distributed along the chain or molecule, such that the molecules are not inserted into, but adsorbed onto the extended surface. Additionally the

insertion of the chain and macromolecules is not very likely just due to their extension.

Regarding the Office's question regarding the solubility of the third amphipathic substance, Applicants again submit that the solubility of the third substance is not particularly relevant to the present invention. In particular, the solubility of the third substance does not play any role for the association of the third substance to the extended surfaces. Solubility is only a criterion for the selection of the extended surface forming substances (first and second substance), which have to differ in their solubility because this difference directly affects the characteristics of the surfaces formed by these substances by the insertion of the more soluble second substance into the surface formed by the less soluble first substance. Thus, solubility of the third substance is irrelevant (as long as dissolution forces are not stronger than the propensity to adsorb to the extended surfaces), as the third substance is not involved in the formation of the surfaces, but only associates thereto. Properties that are important for the selection of the third substance are, for example, charge distribution or the size of the molecule. Therefore, the feature of solubility is not mentioned in the claims, as there is no need for the person skilled in the art to particularly pay attention to the solubility of the third substance. Thus, not mentioning a feature, which is irrelevant to the present invention, can not lead to a lack of clarity.

Regarding the Office's suggestions naming the substances, Applicants have previously defined the substances further as belonging to the classes of "lipids", "edge-active (i.e. surface-active) substances and surfactants", and "chain molecules", respectively. Thus, the substances are named such that the selection of the substances in the present technical field is clear to any one skilled in the art knowing the named substances as surface building substances, surface destabilising substances or substances forming only small surfaces like micelles (as described above in detail). Any further specification of these substances would require inserting

all of the specific substances mentioned in the corresponding subclaims into the base claim, which is unnecessary and unduly restricting the present invention.

Regarding the Office's objections to the terms "less extended" and "more extended" as being indefinite since they are relative terms, Applicants respectfully traverse. Applicants respectfully submit that in view of the explanations herein with regard to less and more extended surfaces and the given components, it is clear to the person skilled in the art, what is meant be more and less extended surfaces. However, Applicants respectfully submit that these objections have been rendered moot by the amendments herein.

Regarding the objection of overlapping substance classes, this has been addressed above with respect to phospholipids belonging to the first and second class, also explaining the basic characteristics of each group regarding substitution, etc.

The Office further asserts that surface active polypeptides, which are classified as second substance, but could also fall under class 3 being chain molecules. Applicants respectfully submit that on page 9, lines 12-16, it is explicitly set out that the terms "adsorbat", etc. (referring to the third substance) are used interchangeably to describe an association between the molecules, which do not form extended surfaces under the conditions chosen. Thus, although polypeptides might be chain molecules, they are clearly not to be classified as the third substance according to the disclosure.

Further, on page 13, lines 7 to 11, it is specified that for the purposes of the present invention, strong anchoring of the adsorbent is excluded, which might result in poorly reversible association. Polypeptides, however, as well as any other representative of the first or second class, exhibiting such strong bondings that they form surface, do not fulfil this requirement and, therefore, are not suitable and intended to act as an adsorbent according to the disclosure.

Regardless, in order to exclude any incidental overlap between the classes, Applicants have amended claim 58 so as to specify that "the at least one first, at least one second and at least one third substance are different from each other."

Regarding the Office's objection to the term "type", this term has been deleted accordingly.

Regarding the Office's assertion that compounds such as sterols in the first class of compounds and sterols by themselves do not form vesicles or extended surfaces, Applicants respectfully submit that certain derivatives of sterols may be modified to be more hydrophilic, e.g. by chemical addition of polyoxyethylene chains to at least one of the polar molecular residues. This increases water solubility of resulting molecules depending on the nature and size of attached moieties, as exemplified by "water soluble tocopherol" (vitamin E-TPGS available from Eastman, see enclosed *Sales Specification*), which enables the molecules to form extended surfaces. These are the sterols envisaged by the present invention when referring to a surface building first substance.

Regarding the Examiner's interpretations of extended surfaces, namely 1) molecules of each class in side by side existence, thus forming an extended surface; and 2) class 2 compound binding to the end of class 1 compound and thus, extending the surface vertically, Applicants respectfully submit that it is well known to one of skill in the art that surface-active substances, as indicated by their name, tend to react with surfaces, e.g. lipid surfaces. This is the principle of dish-washing - destabilisation of fatty acid aggregates on the dish by insertion of surface-active substances into the aggregates and following dissolution of the same. According to the Examiner's second alternative, addition of surface-active substances to the dirty dish would only cause lumps of fat and surfactant on the dish and in the washing water, it would never dissolve fat or fatty acid aggregates.

The same principle applies to the extended surfaces of the present invention. Surface-active molecules are inserted into the lipid surfaces such that the surfaces are destabilised leading to an increase of the extension of the surfaces and of the association capability of the surfaces. However, the amount of surface-active substance must not be to high, such that only destabilisation is achieved, but not complete dissolution of the surface.

More specifically, for example, single chain phospholipids, and other surfactant-like lipids, always insert their hydrophobic chains into, rather than onto, an extended surface, which forms spontaneously upon combining, for example, double-chain phospholipids with water or an aqueous solvent. Single chain phospholipids, thus, insert themselves into, but do not adsorb onto an extended surface of amphiphilic aggregates. This is discussed in the enclosed publication by Hoyrup et al. The bilayer solubilising effect of such insertion is discussed in the paper by Senisterra et al.

The fact that bilayer solubilising amphipaths do not form extended surfaces in a polar solvent is generally accepted by those skilled in the art. This also includes the formation of multilayers as suggested by the Examiner. Such surfaces, which normally correspond to vesicular or lamellar phases for the surfactant like molecules, are only observed in the low water concentration region of phase diagrams, as can be seen from several ternary phase diagrams reported in Almgren.

The mechanism described by Applicants, thus, relies on surfactant insertion, for example, into lipid bilayers, and subsequent chain molecule adsorption to the extended surface of an aggregate comprising at least one surfactant-like amphipath and another surface building amphipath. The article of Almgren describes the mechanism of lipid bilayer solubilization by a number of surfactants and confirms that surfactants do not form superficial layers on top of a lipid bilayer.

Regarding the Examiner's assertion that some of the compounds, i.e. phospholipids, are lipophilic and, therefore, are not water-soluble, Applicants respectfully submit that phospholipids are amphiphilic. Thus, they have lipophilic **and** hydrophilic portions and, therefore, are more or less water-soluble depending on the extent of the hydrophilic portion. Tritons are also water-soluble. In fact, tritons are even more water-soluble than, for example, phospholipids, which also have lipophilic character, which perfectly reflects the selection criterion of the less water-soluble first substance (having extended surface building properties; i.e. typical lipids as named in claim 58) and the more water-soluble second substance (having surface-destabilising properties or being only capable of forming small surfaces, i.e. typical surface-active substances as named in claim 58).

Regarding the Office's suggestion to define each class in terms of specific compounds in the claims and to restructure the claims to clearly indicate what is being claimed by claiming the composition in terms of specific components and name the liquid medium, Applicants respectfully submit that this is unnecessary and unduly restricts the scope of the present invention. As clearly demonstrated herein, the components of the present invention, as well as their selection is clearly defined and enabling a person skilled in the art to carry out the present invention. In particular, one would

- Select the surface building substance, e.g. phosphatidylcholine, check its solubility, (e.g. by looking on the product specification),
- Select a surface-active substance being less soluble in the given medium, e.g. water.
- Mix these substances by adding the more soluble substance, but only to an extent that the surfaces are not dissolved.
- Add the third substance and mix the combination.

Regarding the Examiner's objection to the formulation "substance being less capable of forming extended surfaces" in claim 105, Applicants respectfully disagree. However, Applicants have amended claim 105 to delete this terminology.

The Office further asserts that "Adrenocorticoids and androgens recited in claim 128 are not chain molecules at all." Applicants believe that the Office is referring to claim 126, which indicates adrenocorticoids and androgens. Regarding claim 126, the claim sets out that "the third substance acts as an adrenocorticoid, a ß-adrenolyte, an androgen an antiandrogen, an antiparasite, an anabolic, an anaesthetic,..." Thus, this does not mean that the third substance necessarily is an adrenocorticoid or adrenogen. Rather, it only means that the third substance, which is a chain molecule, acts as (has the same effect as) an adrenocorticoid, an adrenogen, etc. For example, there are some proteins that exert androgen action in biological systems, whether alone or in combination with other active substances.

Regarding the Markush groups in claims 128 (126) and 101, the Office asserts that claim 128 (126) recites improper combination of Markush members because some are specific compounds and others are generic terms and that the case is similar with claim 101. Applicants respectfully traverse. It is well established that:

> The materials set forth in the Markush group ordinarily must belong to a recognized physical or chemical class or to an art-recognized class. However, when the Markush group occurs in a claim reciting a process or a combination (not a single compound), it is sufficient if the members of the group are disclosed in the specification to possess at least one property in common which is mainly responsible for their function in the claimed relationship, and it is clear from their very nature or from the prior art that all of them possess this property. (See MPEP § 2173.05(h))

In this case, claim 101 lists possible surfactants. All of the components listed in claim 101 possess surfactant properties and, thus, possess at least one property in common which is mainly responsible for their function in the claimed relationship. Similarly, claim 126 lists possible components for use as the third substance. All of these components are chain molecules and macromolecules. As set forth, and as is obvious

from the claim, these components are of biological origin and are bio-agents (see page 20, lines 2-4). Accordingly, Applicants respectfully submit that the claims do, in fact, recite proper Markush groups.

Regarding claim 99, which referred to an embodiment wherein the second and third substance are identical, claim 99 has been deleted and, thus, rejection of this claim is moot.

Regarding the Office's question regarding the term "adrenolyte" as recited in claim 126 and 162, this term has been replaced with the term "adrenolytic drug", which related to agents that neutralize the effects of adrenergic substances. Further, the term "rhinologicum" in claim 126 has been replaced with "naso-active drug", which corresponds to the German term "rhinologicum".

Regarding the Office's objection to the term "miniature droplets" in claim 172, as lacking antecedent basis, claim 172 has been made dependent from claim 87, which specifies this term. Regarding the Office's assertion that it would not be clear where the third substance is attached, Applicants have amended the claim accordingly to state that the at least one third substance associates with the exterior surface of said droplet.

According to the Office, the claims indicate that the third substance is on the second substance. However, this is incorrect. The second substance is inserted into the extended surface formed by the first substance. As set forth above, this is the well-known behaviour of surface-active substances when added to extended surfaces formed by a first class substances.

Regarding the Office's objection to claim 191 with regard to "partially amphipathic" substances, this feature has been deleted.

2. 35 U.S.C. §101 Rejections

Claims 186-193 have been rejected under 35 U.S.C. §101 as being a claimed recitation of use without setting forth any steps involved in the process. To expedite prosecution, the claims have been amended as requested.

3. 35 U.S.C. §102 Rejections

WO 92/03122

Claims 58-193 have been rejected under 35 U.S.C. §102(b) as being anticipated by WO 92/03122. The Office asserts that WO 92/03122

> discloses a composition containing two or more amphiphilic substances with different solubilities for the administration of various active substances including insulin.

However, the Office indicates that this rejection will be reconsidered upon the review of an English translation or English equivalent of said document. Applicants enclose herewith an English translation for the Office's review.

Applicants teach a combination of substances in a liquid medium for optimising and controlling the association of substances to extended surfaces. In particular, the combination comprises at least one first surface-building amphipathic substance selected from lipids, lipid-like materials and combinations thereof; at least one second surface-destabilising amphipathic substance selected from surface-active substances, surfactants and combinations thereof; and at least one third amphipathic substance selected from chain molecules and macromolecules. According to Applicants, the first substance and the second substance form extended surfaces in contact with the medium. Further, the solubility of the second amphipathic substance in the liquid medium is greater than the solubility of the first amphipathic substance in the liquid medium. Still further, the molecules of the third substance associate with the extended surfaces formed by the first substance and the second substance. As further set out, the presence of the second substance in the combination increases the ability of the extended surfaces formed by the first substance and the second substance to

associate molecules of the third substance. Finally, the first, second and third substance are different from each other.

As shown in the translation provided, WO 92/03122 does not describe or otherwise suggest the association of macromolecules to extended surfaces, as taught by the present invention. Rather, WO 92/03122 exclusively refers to the incorporation of active agents into the described "transfersomes" and their subsequent transport through natural barriers like the skin. WO 92/03122 does not describe or otherwise suggest the association, i.e. adsorption, of macromolecules specifically at extended surfaces as taught by the present invention. Rather, according to the WO 92/03122 reference, droplets are formed from a solution containing the active agent. This automatically leads to encapsulation of the active agent in the droplet surrounded by the membrane. The agent solution then just forms the "filling" of the vesicle. Since the agent is dissolved, it will not associate with the membrane in any relevant amount. In the present.

It is well-established that the mere fact that a certain result or characteristic may occur or be present in the prior art is not sufficient to establish the inherency of that result or characteristic. *In re Rijckaert*, 9 F.3d 1531, 1534, 28 USPQ2d 1955, 1957 (Fed. Cir. 1993) (reversed rejection because inherency was based on what would result due to optimization of conditions, not what was necessarily present in the prior art); *In re Oelrich*, 666 F.2d 578, 581-82, 212 USPQ 323, 326 (CCPA 1981). "To establish inherency, the extrinsic evidence 'must make clear that the missing descriptive matter is necessarily present in the thing described in the reference, and that it would be so recognized by persons of ordinary skill. Inherency, however, may not be established by probabilities or possibilities. The mere fact that a certain thing may result from a given set of circumstances is not sufficient.' " *In re Robertson*, 169 F.3d 743, 745, 49 USPQ2d 1949, 1950-51 (Fed. Cir. 1999); MPEP 2112.

As provided in MPEP-2131, a claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference. *Verdegal Bros. v. Union Oil Co. of California*, 2 USPQ2d 1051, 1053 (Fed. Cir. 1987).

It is clear that the WO 92/03122 reference does not teach each and every element of the present claims, either expressly or inherently. In particular, WO 92/03122 does not teach a combination of substances (first substance, second substance and third substance) in a liquid medium for optimising and controlling the association of substances to extended surfaces, wherein molecules of the third substance associate with the extended surfaces formed by the first substance and the second substance.

Accordingly, reconsideration and withdrawal of the rejection is respectfully requested.

Uster - 4,944,948

Claims 58-61, 63, 66-76, 91-96, 98, 101, 117, 126-127, 157-171, 174, 186, 187 and 189-192 have been rejected under 35 U.S.C. §012(b) as being anticipated by Uster. The Office asserts that:

> Uster discloses a combination of three amphipathic substances and the formation of vesicles. This combination includes two phospholipids, PC, PG and a macromolecule, EGF. The compositions further contain cholesterol.

Applicants respectfully traverse. The Uster reference relates to a composition and a method for treating wounds or surgical incisions with a sustained release dose of epidermal growth factor (EGF). The combination includes a high-viscosity aqueous suspension consisting of (a) negatively charged EGF liposomes containing neutral phospholipid, negatively charged phospholipids, and liposome entrapped EGF, and (b) means of imparting high composition viscosity such as a low-conductivity aqueous medium containing a zwitterionic compound having an isoelectric point between pH

5.5 and 8.5 and empty liposomes. The composition may further contain cholesterol.

Thus, while the compositions of Uster might contain phospholipids and macromolecules, since these compositions are directed to a high viscosity formulation, they necessarily also contain zwitterionic components such as neutral amino acids and empty liposomes to provide the required viscosity, all of which are not involved in surface formation or association of a third substance in accordance with the teachings of the present invention. In particular, zwitterionic components are neither necessary nor desired for the formation of extended surfaces according to the present invention. For example, neutral amino acids do not fall under the group of surface building substances or under the group of surface destabilising substances according to the present invention. Further, these components are not suitable as associating substance, as they are too small. Even if zwitterionic substances could be considered as third substance (which would require that they actually are capable of associating with the extended surfaces of the present invention) then they would be rendered unsuitable for their intended purpose. In particular, the zwitterionic components of Uster are specifically added for their viscosity imparting characteristics. If they were to be considered to be a third substance in accordance with the present invention, then they would be bound to the surfaces and, as a result could not impart viscosity as required. Applicants respectfully submit Uster does not teach each and every element of Applicants' claims. The amino acids of Uster do not and could not associate with extended surfaces as taught by the present invention. Further, empty liposomes, which are essential in Uster, are not mentioned or desired for use in the present invention.

Further, while Uster mentions phospholipids, the compositions of Uster require a combination of negatively charged and neutral phospholipids in order to provide interaction between negatively charged membrane and positively charged EGF. This is in clear contrast to the combinations of the present invention, which claims the same charge for the surface and third substance associating thereto.

Still further, according to Uster, cholesterol serves the well-defined purposes of increasing the stability of liposomes and reducing the release of EGF. Thus, cholesterol is an integral component of the compositions of Uster, while it is only one of a number of possible membrane forming substances in the present invention.

Finally, Uster does not disclose a more soluble, surface-destabilising component, which is an indispensable component of the combinations of the present invention.

Thus, Applicants respectfully submit that the teachings of the present invention and the compositions of Uster represent completely different systems having some components in common, while differing regarding the most essential components.

Accordingly, the present invention is not anticipated by Uster.

Klibanov

Claims 58-60, 63, 66-74, 91-98, 100-101, 110-122, 151-152, 171-180, 186, 189-192 have been rejected under 35 U.S.C. §102(b) as being anticipated by Klibanov. The Office asserts that

> Klibanov discloses liposomes contianing three amphipathic substances. They incude phospholipids and cholesterol. The third amphipathic substance which is on the surface is either PEG or an antibody.

Applicants respectfully traverse.

The Klibanov reference relates to the activity of amphipathic polyethyleneglycol in prolonging the circulation time of liposomes in the blood. The disclosed liposomes may consist of phosphatidylcholine and cholesterol with PEG-PE or the gangliosid GM_1. In other words, the three amphipathic substances, PEG-PE and GM_1, allegedly correspond to the third substance of the present invention.

However, throughout the entire Klibanov document it is specified that PEG-PE or GM_1 are "incorporated into the membrane". For example, "incorporation of PEG-PE into the liposome membrane" is explicitly mentioned (e.g. p.144, 2nd paragraph) and incorporation of monoclonal antibody into the membrane is explicitly described (e.g. p. 145, last paragraph).

Further, Klibanov relates to the prolongation of the circulation time of liposomes and how far the substances employed for this prolongation are capable of influencing the target binding and retention of the liposomes. However, association, as well as optimisation of the association properties, of macromolecules or chain molecules are not mentioned. Further, Klibanov does not describe or suggest using the more soluble second substance for the optimisation of the association properties of extended surfaces, as taught by Applicants.

Therefore, even if PEG-PE or GM_1 could fall under the class of chain or macromolecules of the present invention, Klibanov only discloses the incorporation of such substances into the membrane, which is not the subject-matter of the present invention. The present invention teaches association of chain and macromolecules onto extended surfaces. Further, Klibanov does not describe or suggest providing a more soluble substance so as to optimise the association properties of the extended surfaces, as taught by the present invention.

For the above reasons, Applicants respectfully submit that the compositions disclosed by Klibanov are different from the combinations of the present invention. More specifically, Klibanov does not describe or suggest each and every element of Applicants' claims. Thus, the present invention is patentable over Klibanov.

4. 35 U.S.C. §103 Rejections

Claims 58-193 have been rejected under 35 U.S.C. §103(a) as being unpatentable over WO 92/03122 in further combination with either Uster (4,944,948) or Klibanov. The Office asserts that:

> The teachings of WO have been discussed above. In essence, WO basically teaches a combination of the three amphiphilic substaces claimed. What is unclear from WO is whether the third substance (macromolecule) is on the surface of the vesicles. Assuming that it is not, attaching a macromolecule on the surface, if that is desired, is deemed obvious to one of ordinary skill in the art since the reference of Uster teaches that when a macromolecule such as EGF can either be encapsulated or attached to the surface of the vesicles with the same release rates (abstract and col. 9, line 29 et seq., col. 10, line 61 et seq.) One of ordinary skill in the art would expect at least similar results. Uster further teaches on col. 4, lines 26-32 that the negative charge on the vesicles enables the EGF molecules to adsorb on the surface of the vesicle. Although Uster does not teach macro molecules other than EGF, WO teaches a variety of macro molecules and it would have been obvious to one of ordinary skill in the art based on Uster's statement that any macromolecule could be attached to the surface of the vesicles, if such is desired and with the expectation of obtaining at least similar results. One of ordinary skill in the art would be motivated to use an amphipathic molecule such as PEG since Klibanov teaches that such a use would increase the circulation time of the vesicles.

Applicants respectfully traverse.

The WO 92/03122 reference describes preparations in the form of minute droplets with a membrane-like coating consisting of one or several layers of amphiphilic substances incorporating agents for the transport through barriers by maximum deformability of said droplets.

As set forth above, the WO 92/03122 reference does not describe or suggest a combination of substances (first substance, second substance and third substance) in a liquid medium for optimising and controlling the association of substances to extended surfaces, wherein molecules of the third substance associate with the extended surfaces formed by the first substance and the second substance. Further,

there is no description in the WO 92/03122 reference regarding improved and controlled association of macromolecules or the addition of surface destabilising substances for these purposes. Further, as set forth above, neither Uster nor Klibanov remedy these deficiencies.

Uster provides sustained release formulations of EGF for the treatment of wounds. The corresponding solution is a high viscosity liposome composition, in which the specific kind of binding of the macromolecule, i.e. whether adsorbed to or entrapped in the liposome, plays no role (see col. 2, l. 30 to 31). Uster is only directed to the liposomal composition providing the sustained release. Binding of the macromolecule or an optimisation thereof is not addressed at all by Uster. Moreover, regarding the charge of the molecules of the compositions, Uster teaches that the liposomes must be negatively charged in order to be capable of binding positively charged EGF. This is in clear contrast to the present invention, which teaches better association between surfaces and associating substance having the same charge. Thus, Uster actually teaches away from the present invention.

Uster does not describe or suggest a third amphipathic substance selected from chain molecules and macromolecules, wherein the presence of the second substance in the combination increases the ability of the extended surfaces formed by the first substance and the second substance to associate molecules of the third substance.

Applicants respectfully submit that by combining WO 92/03122 (relating to the provision of maximum deformability to liposomal formulations for the transport of agents through barriers) with Uster (being directed to high viscosity liposome compositions, which are to remain on the skin as long as possible), a person skilled in the art only learns that according to WO 92/03122, a formulation providing fast release of an agent through skin may contain surface-active substances, whereas according to Uster, formulations, which are to remain as long as possible on the skin

in order to provide sustained release may contain negatively charged phospholipids mixtures as well as cholesterol and empty liposomes.

Thus, the combination of these documents provides nothing other than a guidance as to how to obtain formulations having contrary properties with regard to permeation through the skin and release. However, there is absolutely no teaching how to optimise and control the association of chain or macromolecules to extended surfaces according to the present invention, let alone the employment of surface-active substances for these purposes.

The same applies with respect to Klibanov, which relates to retention times in the blood circulation depending on the incorporation of different substances like PEG-PE or GM_1 into a lipid membrane. Klibanov particularly relates to the prolonged retention time due to said substances and related thereto to the increase of target binding and retention of the liposomes bearing these target specific anstibodies incorporated into the liposome membrane.

Further, Klibanov relates to the prolongation of the circulation time of liposomes and how far the substances employed for this prolongation are capable of influencing the target binding and retention of the liposomes. However, association, as well as optimisation of the association properties, of macromolecules or chain molecules are not mentioned. Further, Klibanov does not describe or suggest using the more soluble second substance for the optimisation of the association properties of extended surfaces, as taught by Applicants.

Klibanov does not describe or suggest a third amphipathic substance selected from chain molecules and macromolecules, wherein the presence of the second substance in the combination increases the ability of the extended surfaces formed by the first substance and the second substance to associate molecules of the third substance. Rather, Klibanov only describes how to incorporate substances like PEG-

PE or GM_1 into a lipid membrane. The present invention, on the other hand, teaches association of chain and macromolecules onto extended surfaces. Further, Applicants respectfully submit that there is no indication regarding how to optimise the association of such molecules onto extended surfaces, let alone the employment of surface-destabilising substances in this regard, which is the subject matter of the present invention.

Further there is absolutely no suggestion or motivation of this teaching in the cited references. Rather, this comes purely from Applicants' teaching.

Thus, the present claims are patentable over WO 92/03122 in further combination with either Uster (4,944,948) or Klibanov. Reconsideration and withdrawal of the rejection is respectfully requested.

CONCLUSION

In light of the above, Applicant respectfully requests early consideration and allowance of the subject application.

Applicants believe that additional fees are not required in connection with the consideration of the within matter. However, if for any reason a fee is required, a fee paid is inadequate or credit is owed for any excess fee paid, you are hereby authorized and requested to charge Deposit Account No. **04-1105.**

Should the Examiner wish to discuss any of the amendments and/or remarks made herein, the undersigned attorney would appreciate the opportunity to do so.

Respectfully submitted,

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Phase behavior of the antineoplastic ether lipid 1-*O*-octadecyl-2-*O*-methyl-glycero-3-phosphocholine

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Abstract

The physicochemical properties of the antineoplastic etherphospholipid 1-*O*-octadecyl-2-*O*-methyl-glycero-3-phosphocholine were examined in the concentration range 1–35% (w/w) lipid, as a function of temperature (range −10°C to 40°C) and of different aqueous solvents by dynamic light scattering, small- and wide-angle X-ray scattering, differential scanning calorimetry and ultrasonic speed measurements. On cooling the lipid dispersion undergoes a phase transition near 6°C, transforming slowly from a micellar into a lamellar gel phase with interdigitating hydrocarbon chains. The lamellar repeat distance is nearly constant over the hydration range 65–90% buffer ($d = 5.09$–5.14 nm). The size of the micelles in terms of the hydrodynamic radius is 3.8 ± 0.1 nm, the polydispersity is low. Their average shape is spherical. The electron density distribution across the micelle gives 2.5 nm for the extension of the hydrocarbon chains and 1.5 nm for the polar moiety. The existence of micelles was verified up to a concentration of 35% lipid. Throughout this concentration range size and shape do not change significantly. The kinetics of formation of the low-temperature phase is slow on cooling, increasing with increasing concentration. Upon heating the phase behavior shows a hysteresis. The extended lamellar organizations start to break down into smaller aggregates near 3°C. The micellar phase is reformed near 20°C.

Key words: Antineoplastic; Ether lipid; Lipid polymorphism; Quasi-elastic light scattering; Small-angle X-ray scattering; X-ray scattering, small-angle

1. Introduction

1-*O*-Alkyl-2-*O*-methyl-glycerophosphocholine (lysolecithin analog, LLA), has long been known as a cytostatic compound acting on various cancer cell lines in vitro [1–3] and in tumor-bearing animals [4]. The mechanisms of action of this compound and of various analogs that have since been synthesized is different from the mechanisms of action of 'classical' cytostatic agents for which DNA is the target. The activity of this compound is mediated indirectly by stimulating cytotoxic properties of macrophages [5,6] and directly due to cytostatic and cytotoxic properties as well as the induction of differentiation in tumor cells. The molecular mechanisms involved remain poorly understood. LLAs were shown to act on membrane-bound enzymes [7] and interfere with phospholipid synthesis [8] and/or protein kinase C activation [9]. Recent results point to LLAs as antiviral drugs with activity against enzymes required for HIV reproduction [10]. Although LLAs have not yet reached the level of clinical application, several of such compounds, including 1-*O*-octadecyl-2-*O*-methyl-glycero-3-phosphocholine (OMGPC), showed promising results in clinical trials [11,12]. Targeting these drugs to distinct parts of the body is a challenging task. Detailed knowledge of the kind of aggregates formed by the compounds either alone or in combination with other lipids or drugs, is of importance for an

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Abbreviations: OMGPC, 2-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine; LLA, lysolecithin analog; PBS, phosphate-buffered saline (saline: sodium chloride); EDTA, ethylenediaminetetraacetic acid; DLS, dynamic light scattering; SAXS, small-angle X-ray scattering; DSC, differential scanning calorimetry; PDDF, pair distance distribution function; $h = (4\pi/\lambda)\sin\theta$ is the scattering vector, λ the wavelength of the Cu-K_α radiation and 2θ the scattering angle; $\Delta\rho(r)$, electron density distribution; R_H, hydrodynamic radius.

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understanding of their behavior in a biological environment, i.e., body fluids (blood, lymph). For example, half-life in the circulation and preferential uptake by distinct organs greatly depend on particle size [13]. In spite of the promise that these compounds hold as anticancer drugs, their physicochemical behavior in aqueous media remains poorly characterized [14,15].

Information about the physicochemical behavior could be useful in the interpretation of biochemical data such as binding, stimulation or tumor activity studies and could facilitate the elucidation of the molecular mechanisms of LLA activity. Other important parameters in this context are heterogeneity after preparation, reproducibility of preparations and stability upon storage.

The aim of this work is to establish the micellar phase boundaries of OMGPC with respect to concentration and temperature and to characterize the individual phases using dynamic light scattering (DLS), X-ray scattering (small- and wide-angle) and complementary techniques.

2. Materials and methods

2.1. Materials

1-*O*-Octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine was synthesized by condensation of racemic 1-*O*-octadecyl-2-*O*-methyl-glycerol with 2-chloro-2-oxo-1,3,2-dioxaphospholane and subsequent treatment of the cyclic phosphate with trimethylamine under conditions similar to those described by Chandrakumar and Hajdu [16,17]. The lipid was chromatographically purified on silica and aluminium oxide. OMGPC was judged to be pure by the appearance of a single spot on the thin-layer chromatogram using the ternary solvent systems $CHCl_3/CH_3OH/H_2O$ (65 : 25 : 4, v/v) and $CHCl_3/CH_3OH/NH_3$ (65:25:5, v/v) in conjunction with iodine, H_2SO_4 and molybdate as visualizing reagents. In addition, a ^{13}C-NMR spectrum was recorded on a Bruker AM-360 FT-NMR spectrometer (solvent: $CHCl_3$). Reagents used for buffer preparation were purchased in the highest obtainable grade from Merck. The lipid concentration was determined by the phosphorus assay [18].

2.2. Sample preparation

Lipid and buffer were prewarmed to 40°C. OMGPC was dispersed in aqueous buffers intermittently vortexing for 10 s and incubating at 40°C till the dispersion appeared optically clear. The sample was left to equilibrate for about 12 h. The quality of the micellar preparations was routinely examined with DLS. The following buffers were used: 10 mM Tris (pH 7.4); PBS (9 mM KH_2PO_4, 30 mM Na_2HPO_4, 150 mM NaCl) (pH 7.2). If not otherwise mentioned Tris was used. Throughout, the concentrations are given in weight percent (% w/w).

2.3. Dynamic light scattering

DLS measurements were carried out at a scattering angle of 90°. The homemade goniometer is equipped with an argon ion laser (Spectra Physics, model 2020-03, 3 W, 514.5 nm) and an ALV-5000 correlator (ALV, Germany). Briefly, as particles move in solution due to their Brownian motion the light scattered by them fluctuates [19]. Thus the time dependence of the scattering intensity, represented by its correlation function, provides information on scatterer motion. If all particles are of the same size, the correlation function is a single exponential function. Its decay is governed by the diffusion coefficient which can be obtained after linearization by a series expansion, a so-called cumulant fit [20]. The first cumulant c_1 gives the z-average of the diffusion coefficient. The diffusion coefficient D is related through the Stokes-Einstein equation $D = kT/6\pi\eta R_H$ to the hydrodynamic radius R_H, the size of an equivalent compact sphere, where k is the Boltzmann constant, T is the absolute temperature and η is the viscosity of the solvent. For a polydisperse sample this quantity is a mean value putting weight on large particles (for a sphere I_{sca} is proportional to R^6). An idea about the width of the distribution is given by the second cumulant c_2, ordinarily the ratio c_2/c_1^2 (polydispersity index) is taken as a measure of polydispersity. Size distributions were obtained through an Laplace inversion of the correlation function [21,22]. The intensity distribution $D_i(r)$ reflects the intensity with which particles of a certain size contribute to the signal (see above). The correlation functions were plotted on a logarithmic scale. There, each particle size leads to a fast decaying sigmoidal curve. The bigger the particles, the bigger the shift to larger correlation times. DLS was used to determine mean size and size distributions.

2.4. Small-angle X-ray scattering

The SAXS experiments were performed on a Kratky compact camera (slit collimation) equipped with a thermostatted sample holder and a linear position sensitive detector (MBraun, PSD-50M). The scattering intensities are smeared by the finite dimensions of the primary beam, slit width and slit length and its wavelength distribution (Cu-K_β part of the radiation). Solvent and sample were measured several times. After normalization, averaging and subtraction of the solvent scattering, the data were corrected for instrumental broadening effects (desmeared) [23–25]. The micellar scatter-

ing curves for low concentrations were interpreted in terms of the corresponding pair distance distribution functions (PDDF). The PDDF represents a histogram of distances inside the particle weighted with the electron density differences and goes to zero at the maximum particle dimension. It is the convolution square (spatial correlation function) of the electron density distribution $\Delta\rho(r)$. The electron density distribution across the micelle was obtained by deconvolution of the PDDF [26–30]. The method employed assumes a centrosymmetrical structure (spherical, cylindrical or lamellar symmetry). It avoids the square-root operation on the intensities and consequently the phase problem. SAXS was used to obtain information on the size of the micelles, on their internal structure (polar/hydrocarbon chain region) and on the kind of phase formed at low temperatures.

2.5. Wide-angle X-ray diffraction

The lipid dispersions were centrifuged into glass capillaries and mounted on a four circle diffractometer with a temperature control unit. X-rays were generated in a Siemens rotating anode unit, monochromatized (graphit monochromator, Cu-K_α radiation) and point collimated onto the sample. The diffraction patterns were recorded on X-ray films (Polaroid 57, NSN 6750-00-079-7395). The film to sample distance was 12.2 ± 0.1 cm.

2.6. Ultrasonic speed measurements

The ultrasonic speed was measured with a Density & Sound Analyzer (DSA 48) from the Anton Paar (Austria). The time required for a short pulse (170 ns) to run through a well defined sample cell (volume 1 ml) is used to calculate the ultrasonic speed with an accuracy of ±1 m/s. The ultrasonic speed depends on the adiabatic compressibility and the density of the sample. The compressibility of solutions is a function of the temperature and of solute properties. Changes of the ultrasonic speed upon changing the temperature were shown to correlate with phase transitions occurring in the sample [31]. Since the changes are very small we used the specific ultrasonic speed $S_{spec} = (S_{solu} - S_{solv})/S_{solv}$. Changes can be visualized best by taking the negative first derivative of the specific ultrasonic speed $-S' = -d(S_{spec})/dT$ and plotting it against the temperature.

2.7. Differential scanning calorimetry

Calorimetric measurements were performed with a Perkin-Elmer differential scanning calorimeter DSC 4 over the temperature range −10 to 40°C. The dispersions were examined after incubation for different times at −10°C at a heating rate of 3 C°/min. Cooling scans were performed at the same rate.

3. Results

3.1. Preparation of micelles

Initially, lipid dispersions were prepared at room temperature. DLS measurements, however, gave correlation functions with a fast and a slow decaying component, indicating two distinct populations of aggregates, i.e., micelles and larger aggregates. The more and larger, the closer to 20°C. The amount of larger aggregates decreases with increasing temperature of preparation and vanishes at 40°C. Therefore, further preparations were carried out at 40°C. The micellar dispersions do not change on storage at room temperature for months. Incubation of the sample at temperatures well above room temperature after storage for comparable times in the refrigerator does not lead to the reformation of clear dispersions, a fraction of the lipid remains insoluble (white flocs).

3.2. Size of the micelles as determined with DLS

The hydrodynamic radius of the micelles extrapolated to zero concentration as determined from the cumulant fit is $R_H = 3.8 \pm 0.1$ nm ($D_0 = 56.1 \cdot 10^{-12}$ m^2 s^{-1}). The micelles are fairly monodisperse in size as indicated by a polydispersity index of 3–6%. R_H does not change significantly at different temperatures (10°C–40°C) and in different solvents (Tris, PBS with physiological sodium chloride concentration as stated in Materials and methods; additionally, physiological sodium chloride solution alone and Tris-buffered

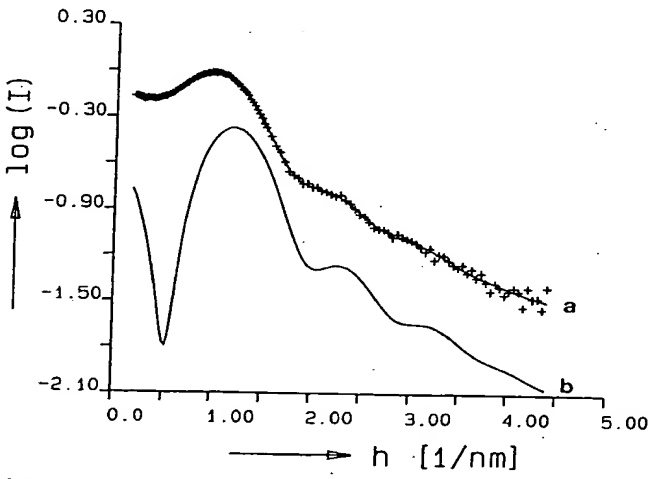


Fig. 1. Curve a: experimental scattering curve of a 1% dispersion at 20°C (crosses) and its approximation function (full line), range 0.177 nm^{-1} ≤ h ≤ 4.404 nm^{-1}. Curve b: desmeared scattering curve.

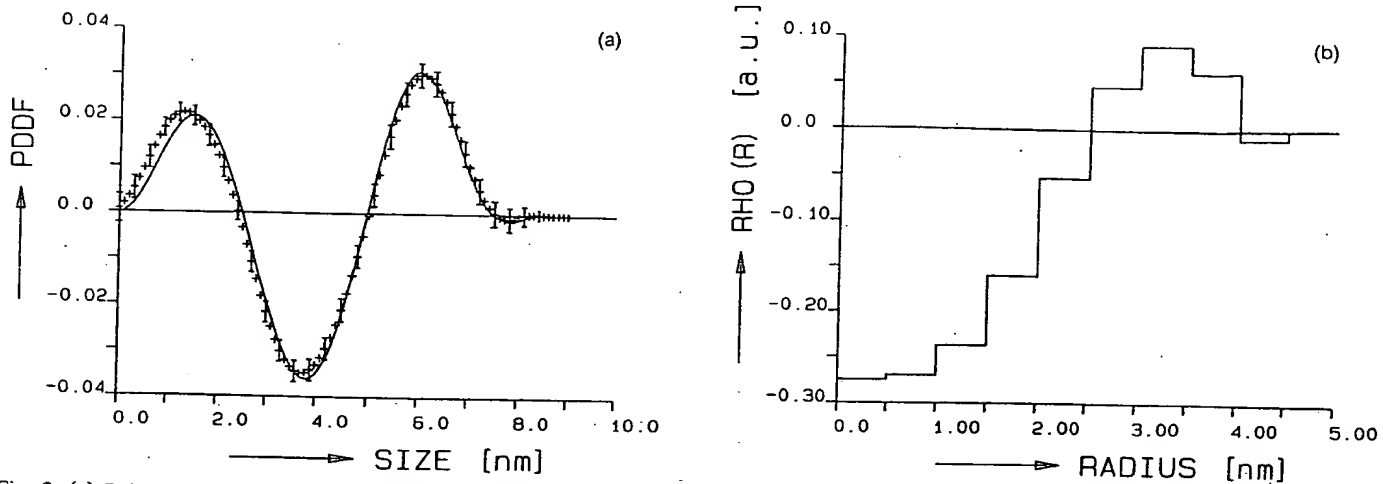


Fig. 2. (a) Pair distance distribution function (crosses) and the fit from the electron density profile (full line). (b) Radial electron density distribution calculated under the assumption of spherical symmetry as a sum of nine equidistant step functions.

potassium chloride (100 mM KCl) with 5 mM EDTA (pH ~ 6) were used).

3.3. Size, shape and internal structure as determined with SAXS

The experimental scattering curve of a 1% dispersion of OMGPC at 20°C (crosses) and its approximation function (full line) are depicted in the range 0.177 $nm^{-1} \leq h \leq 4.404\ nm^{-1}$ in Fig. 1a. The depression of the innermost part of the scattering curve is due to the low overall contrast of the micelles. Fig. 1b shows the desmeared scattering curve. In theory, the scattering curve of a spherical aggregate shows a series of pronounced side maxima separated by zeros. In practice we see only minima because the condition of spherical symmetry is never perfectly fulfilled in real systems. This leads to a smearing out of the maxima and minima.

The same information as in the scattering function, however, in a more straightforward form (real space), is given by its Fourier transform, the PDDF. The PDDF is depicted in Fig. 2a (crosses), the full line is the fit from the electron density profile (Fig. 2b). The maximum dimension of the micelles is approx. 7.7 nm. The negative part is a consequence of the electron density differences across the micelle (polar part and hydrocarbon region). Starting with the PDDF, the best centrosymmetrical electron density distribution $\Delta\rho(r)$ was calculated as a sum of 9 equidistant step functions (Fig. 2b). A brief comment on the meaning of the steps in the electron density distribution in order to avoid its

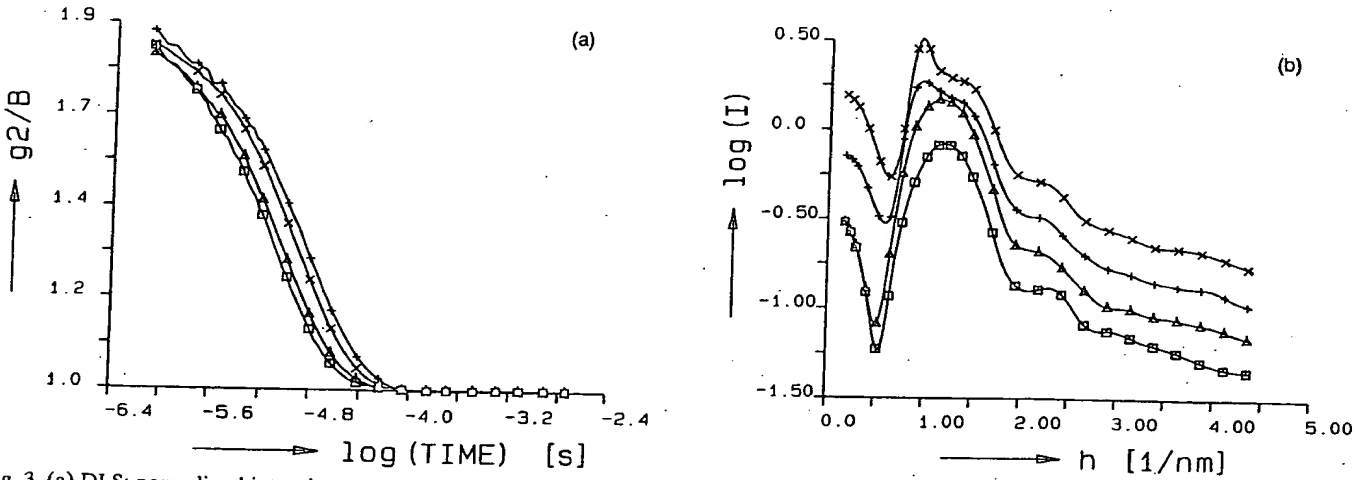


Fig. 3. (a) DLS: normalized intensity correlation functions as a function of concentration (1% (+), 10% (×), 25% (△) and 30% (□)). (b) SAXS: desmeared scattering curves for different OMGPC concentrations shifted by arbitrary factors (1% (□), 10% (△), 25% (+) and 30% (×), $T = 20°C$).

verinterpretation: The step width in the $\Delta\rho(r)$-distribution corresponds to the resolution of the PDDF (i.e., ıe distance of the knots of cubic B-splines). These :eps are not independent, as neighboring steps are orrelated by the stabilization procedure used in the econvolution of the PDDF, i.e., they should not be ıisinterpreted as real steps representing details of the lectron density distribution of the micelles. They just ıodel the $\Delta\rho(r)$-function. A smooth profile running ırough these steps will give an eqivalent fit to the riginal PDDF. For details the reader is referred to ıe original literature [23,24,26–28]. The electron denity distribution gives an idea about the extension of ıe polar region, approx. 1.5 nm, and the hydrocarbon ıoiety, approx. 2.5 nm. This procedure will not be able ɔ fit the PDDF with reasonable accuracy, if the condion of spherical symmetry is not fulfilled. In our case ıe fit to the PDDF (Fig. 2a, full line) demonstrates a ood agreement with the assumption of a spherical ıape. The small deviations in the inner part are due ɔ local electron density fluctuations (background).

4. Aggregation behavior as a function of concentration

The existence of micelles was verified with DLS and AXS up to a concentration of 35% lipid.

(a) DLS. Generally, at higher concentrations the ıotion of each particle is influenced by other particles earby. The intensity correlation functions (Fig. 3a) are ıifted towards smaller correlation times and remain, owever, unchanged in their decay behavior. The apparent diffusion coefficient increases linearly with conentration [32].

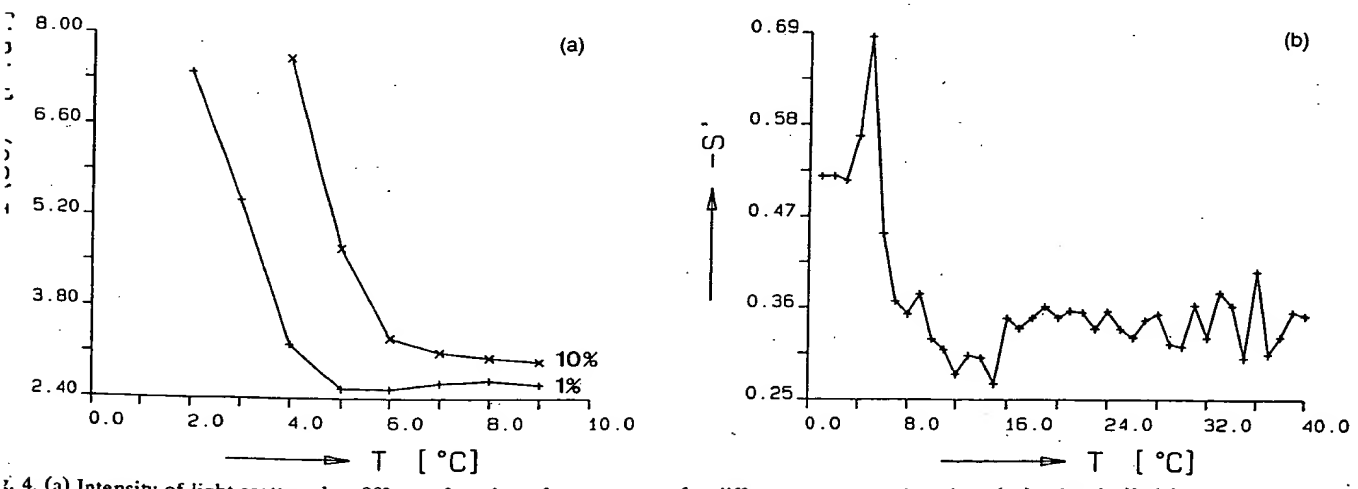
(b) SAXS. In the case of interacting spherical partiles the scattering intensity $I(h)$ can be written as the roduct of the particle form factor $P(h)$ and the structure factor $S(h)$. $S(h)$ describes the interparticle interferences. For dilute solutions it is a constant. The SAXS curves in Fig. 3b show the development of a structure factor with a pronounced maximum for increasing concentrations, superposed on the micellar form factor. The position of the first maximum of the structure factor is related to the interparticle distance. The position and the number of side maxima of the form factor is preserved at all concentrations. This indicates that the micelles do not change significantly in size and shape. Further support of this is gained by the fact that a reasonable structure factor can be obtained by dividing the scattering curve by the form factor. As a first approximation the scattering curve of a 1% dispersion was taken as the form factor. Measurements at lower concentrations are difficult due to the low contrast. A detailed study of the micellar interactions, however, was neither intended nor is it possible on a slit collimating X-ray camera.

3.5. Aggregation behavior as a function of temperature

Phase transitions

Cooling down from 40°C to 0°C, a phase transition occurs near 6°C. This is evidenced by the increase in scattered light intensity at 90° (Fig. 4a) and the change of ultrasonic speed (Fig. 4b). The samples were cooled in 1°C increments with 20 min incubation time at each temperature. Optically, the initially clear dispersions become turbid and jellylike for low concentrations ($< 10\%$) and white and stiff for high concentrations.

DSC experiments were performed to confirm the phase transition. Incubation of a 25% lipid dispersion at −10°C for 30 min gives on heating a small endothermic peak with the maximum at 2.5°C and two sharp endothermic peaks centered near 17 and 20°C (Fig.



g. 4. (a) Intensity of light scattered at 90° as a function of temperature for different concentrations (1% (+), 10% (×)). (b) First derivative of : specific ultrasonic speed as a function of temperature (10%).

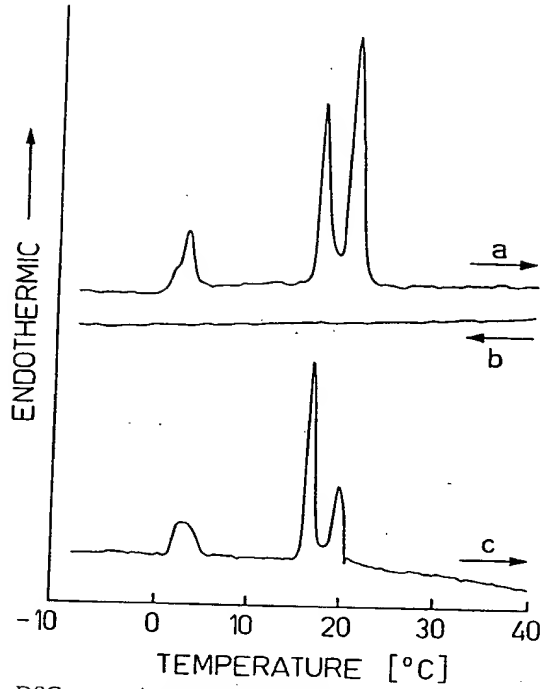


Fig. 5. DSC curves (heating and cooling rate 3 C°/min, 25% lipid). Curve a: heating scan after 30 min incubation at −10°C; curve b: cooling scan; curve c: heating scan after 4 h at −10°C.

5a). The 10% dispersion behaves identically, although with a much smaller low temperature peak. On cooling from 40°C to −10°C at a rate of 3 C°/min, no transition is detected by DSC (Fig. 5b). The 20°C peak decreases on prolonged incubation at −10°C for 4 h (Fig. 5c).

Fig. 6a is a plot of the intensity of light scattered at 90° of a 1% and a 5% dispersion as a function of time at constant temperature ($T = 2$°C). The intensity remains nearly constant for a certain period of time, which depends on the concentration. It is more than 2 h for a concentration of 1%, approx. 1 h for 5% and some minutes for concentrations greater than 20%. During this period only minor growth of the larger aggregates takes place. This initial lag time is followed by a steep increase in intensity, pointing to rapid growth of larger aggregates. The micellar peak decreases at the expense of larger aggregates as shown in Fig. 6b for the 5% lipid dispersion. The formation of the low temperature phase is a kinetically slow process, faster with increasing concentration.

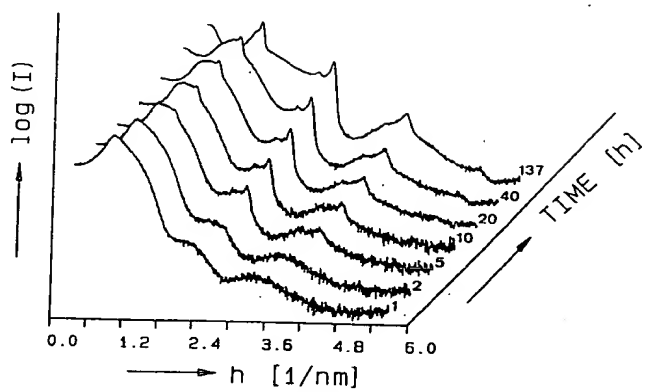
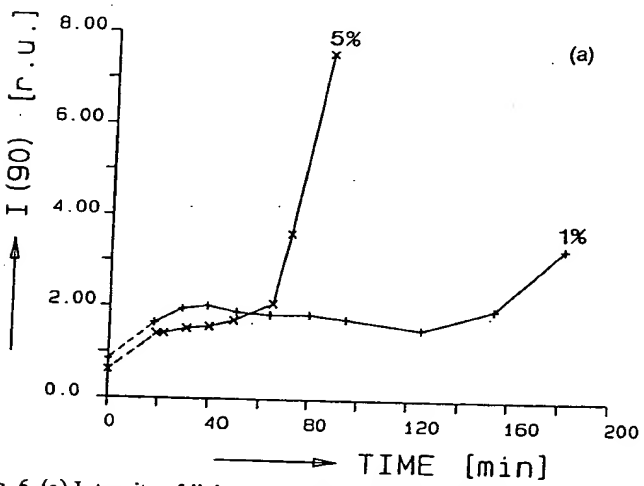


Fig. 7. Experimental scattering curves as a function of time at 0°C shifted by arbitrary factors for better visibility (after 1, 2, 5, 10, 20, 40 and 137 h, $c = 30$%).

Kind of phase formed at low temperatures

The evolution of equidistantly spaced peaks in the experimental SAXS curves (Fig. 7, 30%) proves the lamellar nature of the phase formed. This pattern is representative for concentrations ≥ 10% and for different solvents (see Materials and methods). The curves



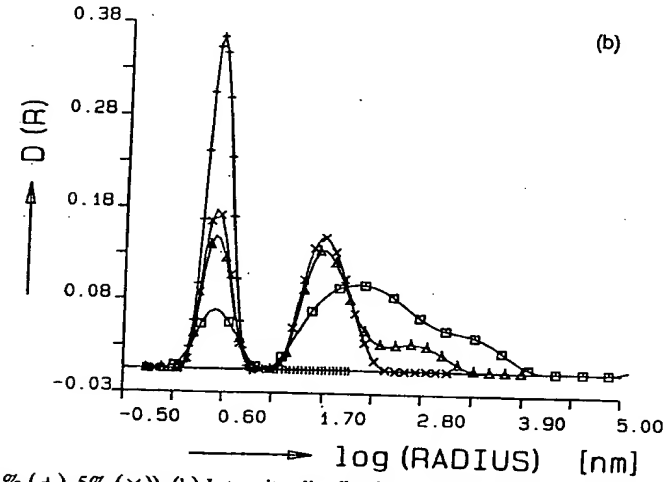
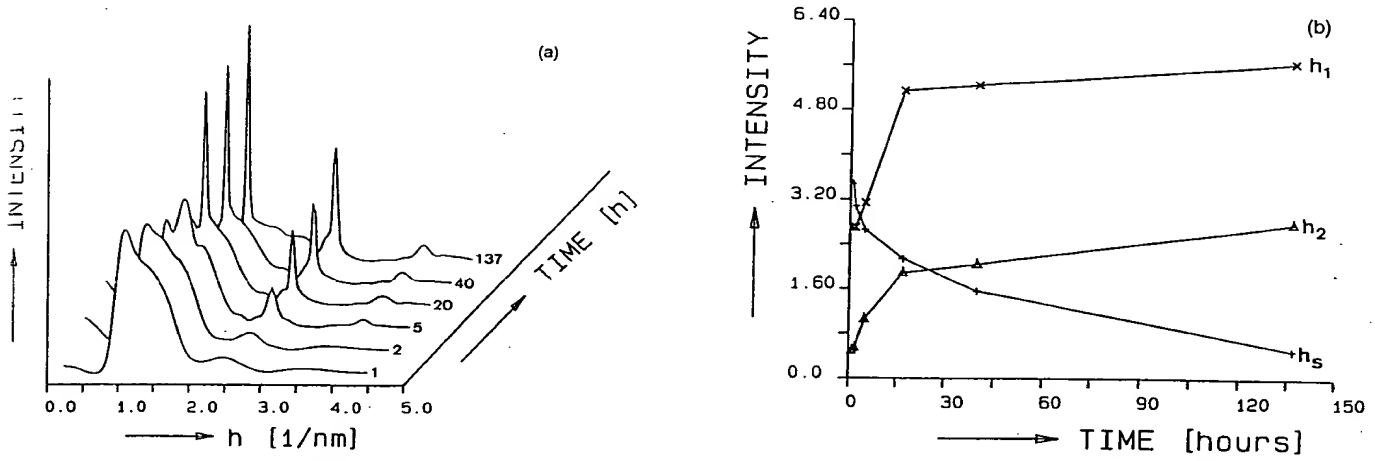


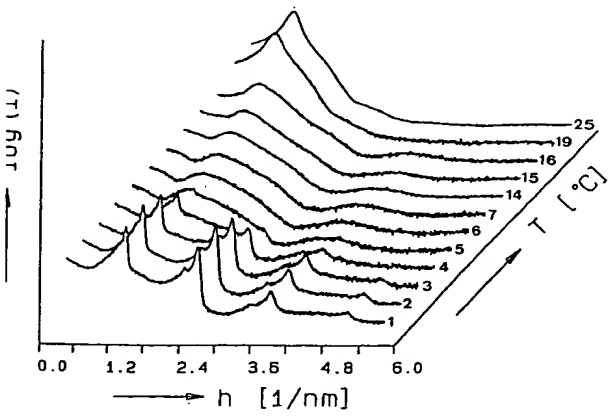
Fig. 6. (a) Intensity of light scattered at 90° as a function of time at 2°C (1% (+), 5% (×)). (b) Intensity distributions corresponding to selected time points of the 5% dispersion in Fig. 6a (1 (+), 20 (×), 40 (△) and 75 min (□)).



ig. 8. (a) Desmeared scattering curves as a function of time at 0°C after 1, 2, 5, 20, 40 and 137 h, $c = 30\%$ (the curves are shifted by an arbitrary actor). (b) Development of the structure factor peak ($h_s = 0.935$ nm^{-1}, (+)) and the 1st (×) and 2nd (△) order lamellar peaks ($h_1 = 1.234$ m^{-1}) of (a) as a function of time.

re smeared by collimation and wavelength effects. he small pre-peaks are due to the K_β line of the -ray radiation. The desmeared scattering curves after , 2, 5, 20, 40 and 137 h at 0°C are depicted in Fig. 8a. he peak of the structure factor of the micelles at = 0.935 nm^{-1} is decreasing at the expense of the first rder lamellar diffraction peak at $h = 1.234$ nm^{-1} (Fig. b). At the same time higher lamellar diffraction or- ers are developing. The lamellar repeat distance d is .09 nm. It remains nearly constant in the hydration ange of 90%–65% ($d = 5.14$–5.09 nm). Again, the onversion of the micellar to the lamellar phase is seen be a slow process. It takes days to weeks to come to ompletion.

Reheating after incubation for several days at 0°C, ie lamellar peaks start to degrade and broaden near °C and transform slowly into a scattering pattern pical for the micellar dispersions near 20°C (Fig. 9).



. 9. Smeared scattering curves upon heating from 0°C to 25°C fted by arbitrary factors (incubation at 0°C for 4 days, 35% lipid persion).

Optical inspection shows that the white gel becomes opaque and eventually clears up near 20°C.

The wide-angle diffraction pattern of a 30% dispersion at −10°C (Fig. 10) shows a single sharp symmetrical ring centered at $(4.3\ \text{Å})^{-1}$. The lipid dispersion was incubated for 4 h, the film was exposed for 14 h.

At concentrations below 10% the low temperature phase behaves rather peculiarly. In the beginning a

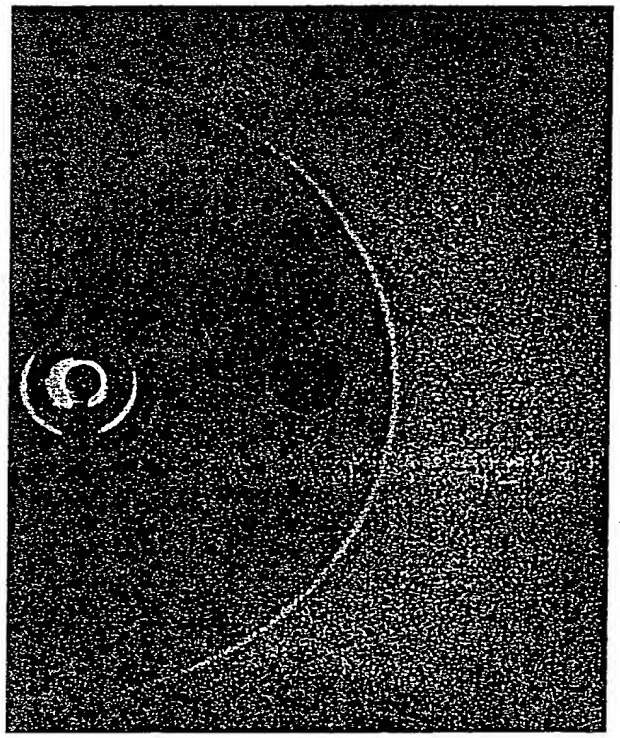


Fig. 10. X-ray wide-angle diffraction pattern of a 30% lipid dispersion at −10°C (incubation time 5 h, exposure time 14 h).

electron density profile gives 2.5 nm for the length of the hydrocarbon chains and 1.5 nm for the polar region.

The phase transition characteristics of OMGPC depend on the thermal and temporal history of the sample. Upon cooling OMGPC undergoes a single kinetically slow transition from a micellar into a lamellar gel phase near 6°C. On heating DSC revealed a hysteresis. The low temperature transition centered at 2.5°C was followed by two additional transitions near 17°C and 20°C. The latter is decreasing with increasing incubation time at low temperatures and represents a metastable phase. Metastable states were also reported for n-octadecylphosphocholine [35] after prolonged incubation at low temperatures and for 1-stearoyl-2-lyso-glycero-3-phosphocholine [37] after too short preincubation at low temperatures. Structurally, the extended bilayers begin to break down upon heating into smaller aggregates, which transform into a micellar phase near 20°C (Fig. 9). The same sequence of heating transitions, lamellar gel below 9.2°C via medium-size vesicles to micelles above 18.4°C, is also reported for 1-*O*-octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine [33,34]. 1-Stearoyl-2-acetyl-*sn*-glycero-3-phosphocholine shows two similar transitions at 6.5°C and 18.5°C [42] corresponding to a lamellar crystalline phase to lamellar gel phase transition (indirect evidence with Raman spectroscopy) and the subsequent formation of micelles. In both cases the behavior on cooling has not been investigated. For n-octadecylphosphocholine [35] the lamellar order started to degrade long before the lamellar to micellar transition indicated by DSC upon heating. The lysolipid in this series, 1-stearoyl-2-lyso-*sn*-glycero-3-phosphocholine, undergoes a single transition at 27°C from a lamellar gel phase to a micellar phase [36,39]. The process of conversion has not been studied. Due to the different methods and measuring protocols employed, the question wether a hysteresis is a general feature of the thermotropic phase behavior of these lipids or not cannot be answered unambiguously.

The low temperature phase is lamellar with a repeat distance of approx. 5.1 nm in excess 'water'. This can be realized only upon interdigitation and/or tilting of the hydrocarbon chains. By argument of geometrical reasons, effective packing of highly asymmetrical lipids, where the projected headgroup area is much larger than that of the tails, is only possible if the chains interdigitate [45]. Chain interdigitation was reported for a series of lysolipids and analogs [33,35,36,39]. In single crystals OMGPC molecules (D-configuration) pack in stacked bilayers with interdigitating head groups and interdigitating and tilting hydrocarbon chains [46]. The wide angle region of OMGPC shows a strong symmetrical ring centered at $(4.3\ \text{Å})^{-1}$. This is indicative for hydrocarbon chains in the gel state. Increasing chain tilt produces an additional more diffuse wide angle reflection following the sharp reflection [47,48]. Our data give evidence that the lamellar phase we see consists of bilayers with interdigitated chains.

The kinetics of formation is slow (days to weeks), faster with increasing concentration. This is a common feature of lysolipids and analogs. DLS experiments (Fig. 6) show an initial lag time where only minor growth takes place, followed by rapid growth. The lag time was the shorter, the higher the concentration. On the other hand, evolution of long range order in the sense of the appearance of diffraction peaks took hours even for higher concentrations (> 10%) (Fig. 7). This behavior was also reported for 1-stearoyl-2-lyso-glycero-3-phosphocholine [36,38]. Wu and Huang [38] described the micellar to lamellar phase transition in terms of nucleation and growth (measurements of fluorescence anisotropy), i.e., (1) formation of lamellar aggregates (hydrocarbon packing rearrangement) and (2) growth of the aggregates to extended lamellar structures.

In dispersions with concentrations lower than 10% a phase separation into a clear micellar dispersion and a cylinder (cylindrical cuvette) occurs after incubation for more than 1 day at temperatures below 6°C. We do not yet have an explanation for this behavior.

Concluding, OMGPC forms spherical micelles which transform into a lamellar gel phase on cooling near 6°C. Reheating, the lamellar gel converts via smaller aggregates into micelles near 20°C. Since OMGPC is widely used in medical and biochemical studies, some practical aspects have to be stressed. For the sake of reproducibility of further experiments, preparations should be carried out at 40°C. Long-term storage of aqueous dispersions in the refrigerator is not recommended.

Acknowledgements

This work was supported by research grant P9214-TEC from the Fonds zur Förderung der Österreichischen Forschung. We thank Doz. Stelzer and his collaborators for the use of their DSC and Doz. Kratky and Karl Gruber for helpful assistance with the wide-angle measurements. In addition, we thank Herbert Stütz for his help with the purification of OMGPC and the determination of the phosphorus content.

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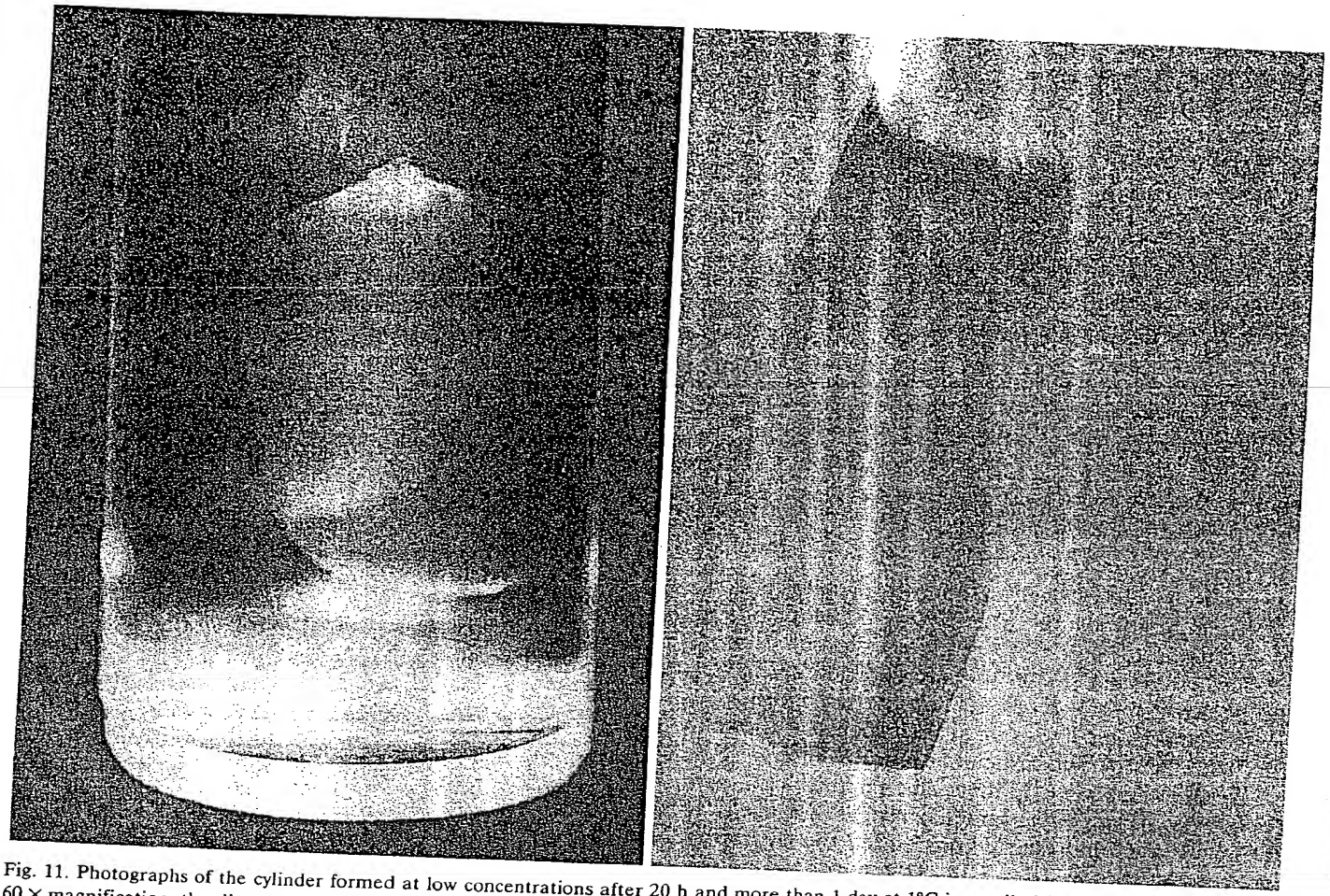

Fig. 11. Photographs of the cylinder formed at low concentrations after 20 h and more than 1 day at 1°C in a cylindrical cuvette (1% dispersion, 60 × magnification, the diameter of the cuvette is 1 cm).

turbid jellylike phase is formed, which is slowly contracting, leaving after more than a day a cylinder (cylindrical cuvette) in a clear supernatant solution. DLS size distributions (not shown) show that the latter is micellar with a small fraction of larger aggregates. Approx. 30% of the total amount of lipid is in the cylinder (phosphorus assay). Fig. 11 shows two photos of the cylinder taken after 20 h (left) and more than 1 day, respectively.

4. Discussion

In spite of their importance as antineoplastic substances and their potential in drug targeting, the physicochemical properties of ether lysolipid analogs have not yet been systematically investigated [15,33–35]. The corresponding ester lysolipids and their analogs have been the focus of more attention [36–42]. A micellar to lamellar phase transition at low temperatures was reported for all these compounds. Sample history was shown to affect not only the kinetics of this transition but also the kind of lamellar phase formed. While this transition and the lamellar phases formed have been studied in some detail, the characterization of the micellar phases was neglected.

Structurally, OMGPC is an amphiphile consisting of a long C_{18}-hydrocarbon chain in position 1 of the glycerol backbone, a short methyl group in position 2 and the phosphocholine headgroup in position 3. The relatively large headgroup together with the highly asymmetrical hydrocarbon tail region gives this molecule a 'cone' shaped appearance. This shape allows OMGPC to pack into micellar geometries [43]. DLS measurements show that the micelles are small and monodisperse. The hydrodynamic radius at infinite dilution is 3.8 ± 0.1 nm. This is in good agreement with a diameter of 7.7 nm from our SAXS data and micellar radii reported for n-octadecylphosphocholine determined with DLS, $R_H = 3.7 \pm 0.2$ nm [44] and 1-stearoyl-2-lyso-glycero-3-phosphocholine from NMR diffusion experiments with $R_H = 3.81$ nm [41]. The

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Phase properties of the aqueous dispersions of *n*-octadecylphosphocholine

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Key words: Phospholipase A_2; Lysophospholipid bilayer; Phase transition

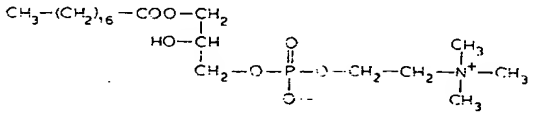
Properties of the aqueous dispersions of *n*-octadecylphosphocholine are examined by differential scanning calorimetry, fluorescence depolarization, light scattering, ^{31}P-NMR, pig pancreatic phospholipase A_2 binding, and X-ray diffraction. On heating, these dispersions exhibit a sharp lamellar to micelle transition at 20.5°C. The lamellar phase consists of frozen (gel-state) alkyl chains which do not bind phospholipase A_2. The kinetics of the transition are asymmetric: the micelle to lamellar transition is very slow and the lamellar to micelle transition is fast. It is suggested that the lamellar phase is a frozen chain bilayer in which the chains interdigitate.

Introduction

Lysophospholipids have been implicated in a variety of biological functions including lysis [1] and fusion [2]. More recently 2-acetyl analogs have been shown to influence the physiological role of platelets, leucocytes, and several other cell types [3], presumably by modulating arachidonate metabolism. The physical properties of their aqueous dispersions are also interesting. For example, the micelles of lysophospholipids are also known to bind phospholipase A_2 [4]. The bilayers formed from an equimolar mixture of lysophospholipids and fatty acids do not bind the enzyme [5].

Lamellar to micelle transitions require a reorganization of the time-averaged shape of the constituent molecules because molecules which pack into planar structures, such as bilayers, do not pack well in highly curved surfaces, such as micelles. However, it has recently been demonstrated that aqueous micellar dispersions of lysophospholipids can form a lamellar phase at low temperatures [6,7]. It was suggested that this phase consists of bilayers in which the acyl chains are highly interdigitated. Since little work has been done on micelle-lamellar transitions in phospholipids, we decided to examine if it occurs with the lysolipid analog *n*-octadecylphosphocholine (Fig. 1). This compound differs from 1-octadecanoyl-

a



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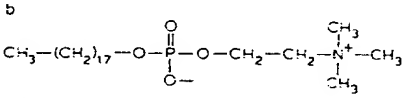

Fig. 1. A comparison of the structures of 1-octadecanoylphosphatidylcholine (a), and the analog *n*-octadecylphosphocholine (b).

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Phospholipid Vesicles

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I. INTRODUCTION

Phospholipid vesicles may be defined as lipid particles surrounded by one or several closed phospholipid bilayers. The space between bilayers and the internal cavity of the vesicle are filled with aqueous solvent. It should be noted that stable or metastable vesicle suspensions require the surrounding phospholipid bilayer to be in the liquid crystalline state. Despite an early start—phospholipids were discovered as early as 1811 [1]—there was not much advance in phospholipid chemistry until the late 1930s. The pioneering discovery of Gorter and Grendel [2] and Danielli and Davson [3] that phospholipid bilayers are fundamental structural elements of biological membranes aroused new interest. Schmitt and his coworkers [4,5] demonstrated for the first time that aqueous dispersions of phospholipids extracted from the myelin sheath give rise to x-ray diffraction patterns very similar to those of intact nerves. The idea of using aqueous phospholipid dispersions as models for biological membranes was born. Bangham and his collaborators showed in the 1960s that "phospholipids spontaneously form closed membrane structures in the presence of water" [6]. They stressed explicitly the usefulness of phospholipid vesicles as models for biological membranes. Most importantly, their work spurred a great deal of research activity devoted to the physicochemical characterization of phospholipids in aqueous dispersions. The final goal of a great number of studies performed by Abrahamsson, Bangham, Chapman, Dawson, de Haas, Luzzati, Saunders, Small, Tanford, Thompson, van Deenen and their coworkers between 1960 and 1980 was to gain insight into the physicochemical properties of cell membranes. As a result of these studies, lipid vesicles (liposomes) have advanced to fundamental tools now widely used in biochemistry, biophysics, and biology.

Phospholipids have been used also commercially for quite some time, e.g., as additives to foodstuffs, emulsifiers, cosmetics, medicinals, lubricants, etc. More recently they have become important as carrier-delivery systems in general and for drug application-targeting in particular. Naturally occuring phospholipids appear to be particularly suited for this purpose, for they are biodegradable and nonantigenic. In the light of the ever-increasing importance of phospholipid vesicles (liposomes), it is not surprising that a flood of original publications, review articles, and monographs on phospholipid

vesicles has appeared in the past ten years. In view of the vast expansion of this field, a comprehensive review on phospholipid vesicles is unrealistic. I shall address my discussion to some points that, to my mind, have not been emphasized appropriately or have even been neglected in the past, and to some recent developments. Furthermore, my discussion will be restricted to vesicles prepared from naturally occurring phospholipids and their synthetic analogs. A discussion of synthetic amphiphiles that form bilayer vesicles but do not occur naturally is beyond the scope of this paper. Such amphiphiles may have important applications, because they can be synthesized with reactive groups that undergo interesting polymerization, polycondensation, and other reactions. By this means, specific vesicle properties may be achieved, such as long-term stability or a specific change in bilayer permeability. The reader interested in this research field is referred to a recent, comprehensive review by Ringsdorf et al. [7] and to Chap. 7 in Part I of this handbook.

II. NOMENCLATURE

According to IUPAC's guidelines, the terms lipid vesicle and liposome should be used synonymously. The following terms and abbreviations are now generally accepted. Multilamellar vesicles or liposomes (MLVs) are defined as spherical particles consisting of concentrically arranged, equally spaced bilayers that are separated by layers of water. MLVs give rise to a characteristic x-ray diffraction pattern comprising a series of sharp reflections in the low-angle region and a broad, diffuse reflection in the wide-angle region. The low-angle pattern consists of a series of sharp reflections in the ratio 1:1/2:1/3:1/4, etc., which is due to the lamellar stacking of the bilayers. The single diffuse wide-angle reflection at 1/4.6 $Å^{-1}$ is due to the lateral packing of the hydrocarbon chains in the liquid crystalline state. Unilamellar vesicles are defined as spherical particles consisting of a single, closed bilayer that surrounds an aqueous cavity. The x-ray diffraction pattern of dilute dispersions of unilamellar vesicles is quite different from that of MLVs. Instead of the series of sharp low-angle reflections, a broad scattering envelope is observed, which by computer simulation can be shown to be the one-dimensional Fourier transform of the electron density profile of a single phospholipid bilayer (see below). Unilamellar vesicles are divided, though somewhat arbitrarily, into two classes according to their size; vesicles with diameters larger than about 100 nm are referred to as large unilamellar vesicles (LUV), vesicles under 100 nm as small unilamellar vesicles (SUV). One justification for this definition of LUV and SUV comes from high-resolution NMR: SUV give rise to a fairly good high-resolution NMR spectrum with chemically shifted resonances, whereas LUV give an unresolved, broad-line NMR spectrum. Still, the line of division is arbitrary, and other authors refer to vesicles below 50 nm in diameter as SUVs and above as LUVs, since packing constraints decrease markedly as the vesicle diameter exceeds about 50 nm [8].

III. LYOTROPIC AND THERMOTROPIC MESOMORPHISM

Phospholipids are amphiphilic, surface-active molecules that have a high tendency to form aggregates both in the dry and in the fully hydrated state. The different kinds of aggregates are termed phases. Generally, lipids are much smaller in weight and size than macromolecules such as proteins, polysaccharides, and ribonucleic acids; yet they are sufficiently large to have distinct regions of greatly different polarity: the hydrophobic

R_H and V increase and pass through a maximum that almost coincides with the micellar phase boundary indicated by the dashed line in Fig. 8a. Dilution of micellar dispersions beyond this boundary yields almost monodisperse SUVs. Vesicle size and dispersity both decrease on the right hand side of the phase boundary. The hydrodynamic radius of the resulting SUVs decreases monotonically with increasing dilution and approaches asymptotically a limiting value of about 15 nm (Fig. 8a). This value is in very good agreement with the average radius of egg phosphatidylcholine SUV produced by detergent removal on Sephadex G-50. Simultaneously, the polydispersity index decreases by ~80% to a limiting value of ~10% at 20-fold dilution (Fig. 8b).

The average size of the homogeneous vesicle population can be varied within limits: both SUV and LUV can be produced with average vesicle sizes ranging from 25 nm to about 200 nm [54,56]. The size and size distribution of these vesicles depend on a number of parameters including the nature of the detergent and lipid used, the lipid-to-detergent ratio, the rate of detergent removal, and experimental conditions such as temperature, pH, ionic strength, and composition of the medium. Unfortunately, our understanding of the effect of these parameters on the size and size distribution of phospholipid vesicles is only qualitative or at best semiquantitative. Most studies concerned with the correlations between the vesicle size and different experimental parameters have suffered from a serious limitation: the important question whether or not the resulting vesicles represent equilibrium structures has not been tackled and hence nothing is known about their thermodynamic stability.

The obvious advantage of monodispersity in terms of well defined vesicle properties (e.g., interfacial area, vesicle volume, etc.) achieved by the detergent removal technqiue is balanced by several disadvantages: (1) with most preparations it is unknown whether or not the resulting vesicles are thermodynamically stable; (2) the encapsulation capacity and efficiency are usually low, in the range 0.2–7 L mol^{-1} and 0.1–15%, respectively; (3) the most serious drawback, however, is the retardation of detergent. Residual detergent affects bilayer properties such as bilayer fluidity (microviscosity) and permeability.

Gel filtration on Sephadex G-50 of mixed cholate-egg phosphatidylcholine micelles generates SUV with a residual cholate level of a few mol % [49]. Less efficient removal of detergent by gel filtration was reported by Allen [53], and this may lead to increased average vesicle sizes. To prevent this, the resultant SUV preparation can be subjected to a second gel filtration or 12 h dialysis; this lowers the residual cholate level to 0.2–0.5 mol % [49].

It is thus clear that the removal of residual detergent is problematic and cumbersome. Current procedures therefore employ detergents such as bile salts and *n*-alkylglucosides that can be removed relatively rapidly and completely due to their high critical micellar concentration. Even though detergent-removal vesicles resemble vesicles produced by other techniques in most physicochemical properties, they still may differ in some properties and thus should be used with caution. For instance, LUVs produced from mixed micelles of egg phosphatidylcholine and 1-*n*-octyl-β-D-glucoside by detegent removal exhibit Na^+, Rb^+, and Cl^- permeabilities comparable with permeability data for sonicated egg phosphatidylcholine vesicles [55]. In contrast, egg phospatidylcholine SUV produced from mixed phospholipid/cholate micelles by cholate removal differ in their surface-chemical properties from SUV produced by sonication; residual cholate confers a negative surface potential, as can be shown by ^{31}P NMR in the presence of lanthanides (H. Hauser, unpublished observation).

V. CHARACTERIZATION OF PHOSPHOLIPID VESICLES

A. Size Analysis

The knowledge of the average vesicle size and size distribution (polydispersity) is essential in biochemical and biophysical studies as well as in commercial applications of phospholipid vesicles. In the following section, a brief assessment of different methods currently employed in the size characterization of these vesicles is given. These methods comprise gel filtration on calibrated columns, electron microscopy, laser-based dynamic light scattering, and analytical ultracentrifugation.

1. Gel Filtration

Gel filtration or gel exclusion chromatography is an inexpensive, convenient method for the fractionation of phospholipid vesicles according to their size and for routine size analyses. For a quantitative analysis of vesicle suspensions, each column has to be calibrated with appropriate standards, such as proteins, virus particles, and monodisperse polystyrene latex beads of known size [57,58]. The method of Ackers has been the method of choice for converting the elution volume V_e to an average hydrodynamic radius R_H according to [59]:

$$R_H = a_0 + b_0 \operatorname{erf}^{-1}(1 - K_D) \quad (1)$$

where a_0 and b_0 are constants characteristic of the column material, and $K_D = (V_e - V_0)/(V_t - V_0)$; V_e is the elution volume; V_0 and $+V_t$ are the column void and total volume, respectively. Gel filtration on calibrated columns provides not only an average hydrodynamic radius but also a semiquantitative measure of the size distribution. The upper limit of the size analysis is determined by the exclusion limit of the column material. Sephacryl S-1000 has an exclusion limit of 200 to 250 nm; this is 3–4 times larger than that of Sepharose 4B. It is the column material with the largest exclusion limit presently available. The usefulness and applicability of Sephacryl S-1000 to the fractionation of polydisperse phospholipid vesicles and the routine size analysis of these vesicles were first pointed out by Tanford and coworkers [66].

Vesicles smaller than the exclusion limit of the column material are fractionated, and from their elution volume V_e the average hydrodynamic radius R_H of the vesicle population is derived from Eq. (1). All vesicles with diameters exceeding the exclusion limit of the column are no longer fractionated but eluted at the column void volume V_0. The choice of column material is therefore important: it determines not only the size range of vesicles that can be analyzed but also the size range of maximum resolution. Gel filtration is very well suited for the fractionation and size analysis of SUVs and LUVs up to diameters of ~250 nm and for the separation of MLVs from SUVs. One obvious disadvantage of this method is its size limitation: vesicles larger than ~250 nm cannot be analyzed with column materials presently available. Vesicles of diameter >1 μm usually fail to enter the gel and accumulate on top of the column. Large vesicles trapped on top of the column may either clog the column or cause serious disturbances in gel filtration. Some of the disturbances arise from chemical degradation of the trapped lipids. Therefore samples for gel filtration have to be freed of large vesicles and particles. Another disadvantage is the nonspecific absorption of lipids and proteins to the column material. In order to minimize absorptive effects the column has to be presaturated with the compound(s) to be chromatographed [58].

2. Electron Microscopy

Electron microscopy has become a popular method for the size analysis of phospholipid vesicles. Its major advantage is that individual vesicles can be viewed directly. It yields a good estimate of size range and the size distribution of phospholipid vesicles, provided that the following conditions are fulfilled. (1) In order to produce visible images of phospholipid vesicles by electron microscopy, the aqueous dispersion of lipid vesicles has to be subjected to cryofixation or chemical fixation and staining. Discussing this methodology in detail is beyond the scope of this review. It suffices to say that these procedures may produce artefacts. For instance, the method of negative staining, though quick and extremely simple, is troubled by its inherent probability of generating artificial lipid structures, particles, phases. This has been clearly demonstrated for negatively charged phosphatidylserine dispersions [21,60]. Details derived from x-ray diffraction were found to be in good agreement with structural data derived from electron microscopy of freeze-fractured phosphatidylserine dispersions. However, by comparison of the results obtained by x-ray diffraction with electron microscopy data of negatively stained samples, it was clear that negative staining of phosphatidylserine dispersions can lead to structural artefacts. The general consensus evolving now is that cryofixation methods, such as freeze-fracturing, freeze-etching, and various modifications thereof, e.g., spray-freezing, freeze-drying, etc., preserve the native structure, whereas all kinds of staining procedures have a great potential of inducing structural changes and in turn artefacts. Therefore results obtained by electron microscopy of negatively or positively stained samples have to be treated cautiously and should be counterchecked by independent methods.

A number of different cryofixation procedures are in use, and also different methods exist for the preparation of surface replicas and shadowing. Some of the details are described in Refs. 25, 36, and 61–63. These procedures yield images that may differ in detail. For instance, the image analysis of samples prepared by ordinary freeze-fracture methods is hampered because the position of the fracture plane is unknown. If the angle of shadowing is 45° with respect to the fracture plane, only vesicles fractured equatorially yield circular images that are shadowed to 50%; the boundary between the light and the dark (shadowed) zone then dissects the circular image. This is important, as only these vesicles give the correct vesicle diameter and should be considered in the size analysis. A procedure for deriving the true vesicle size from freeze-fracture electron microscopy has been reported recently. It is based on the assumption that unilamellar vesicles are fractured randomly [65]. In freeze-dried samples [62] that are not subjected to any fracturing processes, whole vesicles are imaged, and the problem just discussed does not appear [66].

Perhaps even more powerful is the technique of cryoelectron microscopy, which uses specimens embedded in ultrathin layers of ice (~100 nm). This method recently introduced requires no shadowing (staining) and has a resolution of ~3 nm [64,70].

(2) Even if artefacts during the sample preparation are avoided, a good estimate of the average size distribution requires unbiased sampling: electron micrographs have to be taken randomly, and the size analysis has to be based on a representative fraction of vesicles. The latter requirement necessitates the analysis of a sufficiently large number of images. A respectable size analysis should be based on no less than 400 to 500 vesicles. This number determines the significance and accuracy of the analysis, for instance, measuring 400 images of vesicles ($n = 400$) yields an average vesicle size with an accuracy of $(100/\sqrt{n})\% = 5\%$.

(3) The size analysis by electron microscopy is based on the assumption that phospholipid vesicles are spherical. This is by and large correct. Some deviations from strict spherical geometry can be tolerated, and methods of approximation for the size analysis of ellipsoidal particles have been worked out [63].

From the above discussion it is clear that a reliable size analysis by electron microscopy is tedious and time-consuming. It requires costly instrumentation and an experienced operator. This clearly limits the use of electron microscopy as a routine method for sizing lipid vesicles.

3. Dynamic Light Scattering

In contrast to electron microscopy, laser light scattering is quick and easy to perform. The only stringent requirement on the lipid dispersion to be analyzed is the removal of dust particles. Because of the dependence of the scattering intensity on the square of the molecular or particle weight, light scattering is particularly sensitive to the presence of large particles such as dust. Removal of dust particles is achieved either by subjecting the sample in the scattering cell to centrifugation or by using a closed loop filtration system that filters the lipid dispersion continuously into the sample cell. The particle size analysis by light scattering should be carried out with dilute lipid dispersions, usually at lipid concentrations below 1 mg/mL in order to eliminate multiple scattering effects. Modern equipment for the size analysis of phospholipid vesicles is commercially available from several manufacturers (for a thorough discussion of the instrumentation consult Ref. 67).

Light scattering is applied to advantage to unimodal vesicle populations. Dynamic light scattering then can be performed at a single scattering angle Θ (usually $\Theta = 90°$), and the method yields a value for the average hydrodynamic radius R_H in a few minutes. Therefore for homogeneous lipid vesicles light scattering is the most convenient method of size analysis and suitable for routine use. If, however, no *a priori* knowledge of the size distribution is available, the size analysis by light scattering is less straightforward. In this case the intensity autocorrelation function should be determined for different values of both the scattering angle Θ and the correlator sample time [68]. A cumulant analysis of the experimentally obtained autocorrelation function yields a z average hydrodynamic radius $R_H(z)$ and an estimate of the polydispersity. Due to the M_r^2 dependence of the scattering intensity, even the presence of a very small quantity of large particles would produce a significant increase in the average vesicle size. Therefore dynamic light scattering applied to polydisperse phospholipid dispersions may give misleading results, or at least results inconsistent with other methods. However, methods of analysis have advanced significantly in recent years. The data analysis can be greatly improved by the use of the inverse Laplace transformation of the autocorrelation function as discussed in more detail in Refs. 68 and 69. Using this treatment, it is now possible to derive a reliable and accurate estimate of the size distribution of phospholipid vesicles.

Static light scattering merits some comments. It can provide useful additional information pertaining to vesicle size and shape and size distribution. Static light scattering yields a weight average molecular (particle) weight and the z average mean square radius of gyration $\langle R_g^2 \rangle_z$. Since macromolecules and particles of different shape have different R_g values for a given hydrodynamic radius R_H, the radius of gyration contains shape information. For instance, for a multilamellar vesicle that to a first approximation may be

treated as a solid sphere, we have $R_g = (3/5)^{1/2}R_H$, while for LUVs with the bilayer thickness $d << R_H$, $R_g \approx R_H$. Hence a combination of static and dynamic light scattering measurements may yield information as to the lamellarity of the lipid vesicles. The information on vesicle size, shape, and size distribution derived from static light scattering is very precise for large vesicles with a diameter exceeding about 200 nm.

In the following paragraph a few examples of the size analysis of phospholipid dispersions are given, and results obtained with different methods are compared. Egg phosphatidylcholine SUVs were prepared by cholate dialysis of egg phosphatidylcholine/cholate mixed micelles (mole ratio = 0.5, total lipid concentration 39 mM) in 0.02 M Tris buffer pH 7.4, 70 mM NaCl. For comparison, egg phosphatidylcholine SUVs were also prepared by multiple extrusion (10 times) of MLVs dispersed in the same buffer through two stacked membrane filters with a mean pore size of 50 nm [68]. A standard cumulant analysis of the dynamic light scattering data yield an average hydrodynamic radius of R_H = 26.4 nm and R_H = 34.1 nm for the two SUV preparations, respectively. The variances V as a measure of the polydispersity of the two SUV preparations are 5% and 11%, respectively. Thus dynamic light scattering of the two vesicle dispersions indicates that SUVs prepared by the detergent removal technique are practically mono-disperse, with a mean radius of 26 nm, while the SUV preparation extruded ten times through a 50 nm pore size filter is more inhomogeneous in size. Furthermore, the mean hydrodynamic diameter of 68 nm of the latter vesicles exceeds the mean pore size of the filter used. Figure 9 shows the size distribution of these two SUV preparations obtained by inverse Laplace transformation of the intensity autocorrelation function. Vesicles prepared by detergent removal are characterized by a narrow, almost symmetrical size distribution (Fig. 9a), while SUVs made by extrusion yield a broader, asymmetric size distribution (Fig. 9b) tailing off at the side of large vesicles. The maxima in the size distribution are consistent with the average hydrodynamic radii.

For comparison, freeze-fractured samples of the extruded vesicles were prepared, and the bar histogram of Fig. 9c was derived from electron micrographs of freeze-fractured preparations. The number distribution represented by the bar histogram (Fig. 9c) is shifted to smaller sizes compared to the intensity distribution derived from light scattering using the Laplace inversion (Fig. 9c). The maximum in the number size distribution is between 20 and 25 nm, which is significantly smaller than the average hydrodynamic radius R_H = 34.1 nm derived from dynamic light scattering. The reason for the discrepancy is that dynamic light scattering yields the z average of the hydrodynamic radius, whereas electron microscopy gives the number distribution. The number distribution can be calculated from the intensity distribution by taking into account the weighting of the scattered intensity [68]. As can be seen form Fig. 9c, the number distribution derived from light scattering is quite narrow, with a peak at 23 nm. This is in very good agreement with the bar histogram derived from electron micrscopy. Figure 9c illustrates convincingly how strong the affect of a very small number of large vesicles can be on the intensity distribution. The contribution of a small number of large particles is apparently amplified in the dynamic light scattering experiment due to the weighting of the scattered intensity. After correction for this effect, the average vesicle diameter is 46 nm, slightly smaller than the nominal pore size of 50 nm of the membrane filter used for extrusion. When comparing size parameters derived from different methods of analysis, it should be borne in mind that different methods yield different averages for the size parameters such as hydrodynamic radius, vesicle volume, vesicle weight, etc.

Almost all standard preparative methods for the preparation of phospholipid vesicles produce polydisperse phospholipid vesicles. It is clear from the examples cited that only a combination of several analytical methods can provide reliable information about the vesicle size distribution in such populations. A polydisperse vesicle population can be fractionated by gel filtration prior to analysis by dynamic light scattering. This gives more reliable results than the direct application of dynamic light scattering, even at the most sophisticated level. It was shown previously [58] that satisfactory results can be obtained with even highly polydisperse phospholipid vesicles by a combination of gel filtration and dynamic light scattering techniques. Heterogeneous vesicle dispersions were first fractionated by gel filtration on appropriate columns (Sepharose 4B or Sephacryl S-1000), and fractions thus obtained were subsequently analyzed by dynamic light scattering. The mean hydrodynamic radius derived from dynamic light scattering of individual column fractions is in good agreement with the values derived from gel filtration and freeze-fracture electron microscopy [58].

4. Analytical Ultracentrifugation

Analytical ultracentrifugation has been used, though much less frequently, for the size analysis of phospholipid vesicles. It should be the method of choice if the vesicle weight rather than the vesicle size is required. The method was first applied to sonicated egg phosphatidylcholine dispersions by Saunders et al. [29]. Subsequently it was employed in the weight analysis of phospholipid dispersions by several groups [43,49,56, 71–73] either as the sedimentation/diffusion (s/D) or the sedimentation equilibrium method.

The (s/D) method involves the determination of both the sedimentation and the diffusion coefficient at infinite dilution in two series of separate measurements. The vesicle weight M_r is calculated using the Svedberg equation:

$$M_r = \frac{RTs_o}{D_o(1 - \bar{\nu}\rho)} \tag{2}$$

where M_r is the anhydrous vesicle weight, s_o and D_0 are the sedimentation and diffusion coefficients, respectively, extrapolated to infinite dilution, $\bar{\nu}$ is the partial specific volume of the phospholipid, and ρ is the density of the solution. For monodisperse samples the (s/D) method gives vesicle weights in good agreement with the values derived from the sedimentation equilibrium (cf. results compiled in Table 1). The (s/D) method is advantageous when applied to bimodal or paucimodal lipid dispersions. This was shown for unilamellar phospholipid vesicles with integral membrane proteins incorporated in the phospholipid bilayer. Such proteoliposomes differing in the number of integral membrane protein molecules per lipid vesicle can be separated in a single sedimentation velocity run if the molecular weight of the integral membrane protein is sufficiently large [49]. The vesicle weight of each vesicle species can then be determined, provided that the corresponding diffusion data are available. Schlieren optics are the preferred optical system to be used in the (s/D) method; they are particularly useful for bi- and paucimodal dispersions. However, with polydisperse vesicle populations, the (s/D) method is inferior to the sedimentation equilibrium technique. With polydis-

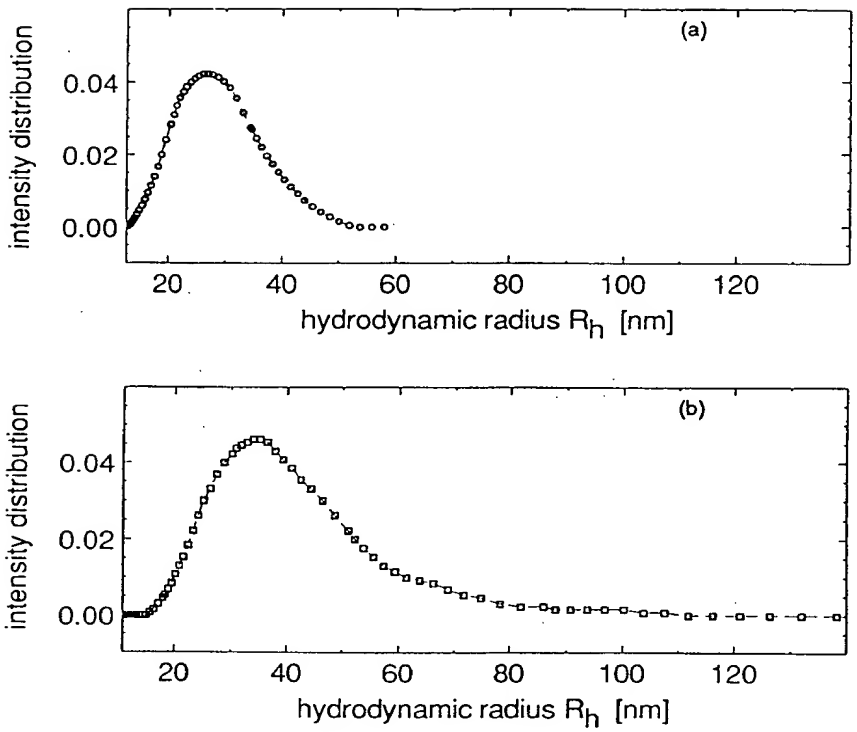

Figure 9 Size distribution of SUV of egg phosphatidylcholine derived from dynamic light scattering. The intensity distributions shown were obtained by inverse Laplace transformation of the intensity autocorrelation functions. (a) Egg phosphatidylcholine SUVs were prepared by cholate removal by dialysis from egg phosphatidylcholine/cholate mixed micelles (mole ratio = 0.5, total lipid concentration 39 mM) dispersed in 0.02 M Tris buffer pH 7.4, 0.07 M NaCl. (b) For comparison, egg phosphatidylcholine was dispersed in the same buffer to a final concentration of 13 mM, and the resulting MLVs were homogenized by multiple extrusion (10×) through two stacked membrane filters of mean pore size 50 nm using a commercially available filtering device (the Extruder ™ from Lipex Biomembranes). (c) The intensity distribution of the sample described under (b) (○) is compared to the number distribution (●) computed from the intensity distribution as described in Ref. 68 and the number distribution (bar histogram) derived from electron microscopy of freeze-fractured samples of the same dispersion.

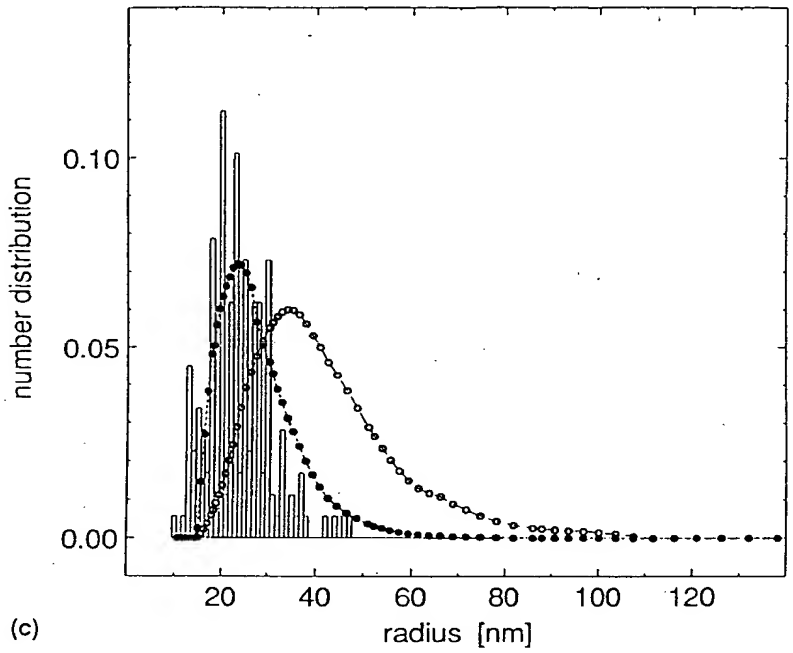

Table 1 Vesicle Weight Determination by Analytical Ultracentrifugation

Phospholipid[a]	Ultracentrifugal method	Vesicle weight (Da)	Calculated[c] R (nm)
egg PC	(s/D)	2.5×10^6	10.5
DMPC	(s/D)	2.33×10^6	
DOPC	(s/D)	2.72×10^6	
egg PC[b]	sedimentation equilibrium	2.66×10^6	10.8
DMPC	sedimentation equilibrium	2.33×10^6	
DOPC	sedimentation equilibrium	2.66×10^6	
egg PC	sedimentation equilibrium	2.70×10^6	10.8
egg PC	approach to equilibrium	range: $2.1 - 4.5 \times 10^6$	9.8–13.4
		average: 3.2×10^6	11.6

[a]All phospholipids were dispersed in 0.01M Tris buffer pH 7.3 containing 0.1 M NaCl and 0.02% NaN_3 and sonicated in order to produce SUV (experimental details are given in Refs. 49, 72, 73). Abbreviations: PC, phosphatidylcholine; DMPC, 1,2-dimrystoyl-*sn*-phosphatidylcholine; DOPC, 1,2-dioleoyl-sn-phosphatidylcholine.

[b]SUV of egg phosphatidylcholine dispersed in the same buffer were made by the cholate removal method (egg phosphatidylcholine/cholate = 0.35–0.5, mole ratio; final phospholipid concentration = 0.013–0.040 M).

[c]Anhydrous radius R calculated from the anhydrous vesicle weight M_r, the partial specific volume $\bar{\nu}$ of the phospholipid, and the anhydrous bilayer thickness d using Eq. (4).

perse samples, the (s/D) method yields a mixed average vesicle weight that is difficult to interpret.

The sedimentation equilibrium method has the advantage of a secure theoretical background with only a few assumptions being involved. Sedimentation equilibrium methods are not only simpler experimentally but also more accurate. Furthermore, for polydisperse vesicle populations, sedimentation equilibrium methods have the advantage of yielding a well-defined weight-average vesicle weight and a good assessment of the polydispersity. From a single sedimentation equilibrium run the weight-average vesicle weight can be determined at each point in the cell, and in addition to these "point" averages, the weight and z average vesicle weight averaged over the total vesicle population are obtained. In sedimentation equilibrium, the anhydrous vesicle weight M_r is derived from the measurement of the solute concentration throughout the cell under conditions of thermodynamic equilibrium:

$$M_r(1 - \bar{\nu}\rho) = \frac{2RT}{\omega^2} \frac{d \ln c}{dr^2} \tag{3}$$

Where ω is the angular velocity in radians/s, $d \ln c/dr^2$ is the slope of the plot of logarithm of lipid concentration ($\ln c$) versus the square of the radial distance (r^2), and other terms have their usual meanings. Since the solute concentration is required as a function of the radial distance r, interference optics and UV absorption are the preferred optical systems. For a homogeneous vesicle population, the plot according to Eq. (3) yields a straight line.

Analytical ultracentrifugation has been used successfully to determine the vesicle weight of egg phosphatidylcholine dispersions produced by sonication [29,43,71,72] and detergent removal [43]. Some representative results are summarized in Table 1. The values for the vesicle weight of sonicated dispersions of different phosphatidylcholines determined by the (s/D) method range between 2.3 and 2.7 $\times$ 10^6 (Table 1). These values agree very well with the results obtained with the sedimentation equilibrium method.

In order to assess the spread in size of sonicated egg phosphatidylcholine dispersions, 12 preparations were analyzed by the approach-to-equilibrium method (or Archibald method). The values for the vesicle weights thus obtained range from 2.1 $\times$ 10^6 to 4.5 $\times$ 10^6 with an average vesicle weight of 3.2 $\times$ 10^6. For comparison, the average vesicle weight of egg phosphatidylcholine SUVs produced by cholate removal was determined by the sedimentation equilibrium method, and a value of 2.66 $\times$ 10^6 was obtained (Table 1). In the last column of Table 1, vesicle radii are listed, calculated from the anhydrous vesicle weight M_r using the equation

$$R = \frac{d}{2} \pm \frac{[(d^2 - 4(d^2/3 - M_r\bar{\nu}/4\pi Nd)]^{1/2}}{2} \tag{4}$$

where d is the thickness of the anhydrous bilayer and N is the Avogadro constant. R depends on the choice of d. For the calculation of R values included in Table 1, a d value of 4.78 nm [74,75] and a value of $\bar{\nu}$ = 0.9839 cm^3/g [72] typical for egg phosphatidylcholine was used. This d value is based on x-ray diffraction studies of McIntosh and Simon [74], who determined the P-P distance from electron density profiles of egg phosphatidylcholine as 3.78 $\pm$ 0.08 nm. Lesslauer et al. [75] have shown the high-density peak in the electron density profile to be close to the center of the phosphatidylcholine head group. From space-filling molecular models, the distance from the center to the outer edge of the phosphatidylcholine head group is taken as 0.5 nm. Therefore the d value of the

anhydrous egg phosphatidylcholine bilayer is obtained by adding 1.0 nm to the P-P distance, yielding $d = 4.78$ nm. The anhydrous vesicle radii calculated from Eq. (4) are in satisfactory agreement with experimental values derived from hydrodynamic measurements. For instance, diffusion measurements at 20°C on sonicated egg phosphatidylcholine dispersions yield a value for the diffusion constant $D_0 = 1.80 \pm 0.07 \times 10^{-7}$ cm^2/s, which is consistent with a Stokes radius of $R_H = 12.0 \pm 0.5$ nm [72]. The Stokes radius can be identified with the outer radius of the hydrated phospholipid vesicle; it is obtained from the diffusion coefficient D_0 using the Stokes-Einstein equation

$$R_H = \frac{kT}{6\pi\eta D_o} \tag{5}$$

where η is the viscosity of the suspending medium.

Gel filtration on Sepharose 4B and electron microscopy of the same dispersion of egg phosphatidylcholine SUV give average values for the hydrodynamic radius of 13.1 nm and 12.9 nm, respectively [72]. The hydrodynamic radius of egg phosphatidylcholine SUVs prepared by the cholate removal method was determined by gel filtration and freeze-fracture electron microscopy, and the values thus obtained are 10.5 and 13.5, respectively. As expected, the hydrodynamic radii R_H are greater than the "anhydrous" radii R (Table 1) calculated from Eq. (4).

Despite many advantages, analytical ultracentrifugation has not become a routine method for the weight (size) analysis of phospholipid vesicles. The reason for this is that the equipment required is rather elaborate and relatively expensive compared to the instrumentation needed to perform gel filtration and dynamic light scattering measurements. Moreover, sedimentation equilibrium runs are time-consuming, and even with short sample columns and the overspeeding technique the weight determination of phospholipid vesicles normally takes 1–2 days [73].

B. Lamellarity

A qualitative indication of the lamellarity of phospholipid vesicles is provided by freeze-fracture electron microscopy. Cross-fractures of MLVs reveal the concentric stacking of bilayers. A somewhat better estimate of the lamellarity of phospholipid MLVs is derived from ^{31}P NMR in the presence and absence of shift or broadening reagents. For instance, in the presence of impermeable, paramagnetic broadening reagents such as the transition metal ions Mn^{2+} and Gd^{3+}, the intensity of the ^{31}P NMR resonance is reduced. The reduction in the signal intensity is directly proportional to the fraction of phospholipid present in the outer monolayer of the external bilayer exposed to the broadening reagent. This fraction will be very small for highly multilamellar vesicles, but it approaches unity for LUVs. The approach using paramagnetic probes and also specific labeling methods based on impermeable reagents still yield only an approximate estimate of the lamellarity. This is due to packing defects present in MLVs that make internal lamellae of MLVs accessible to ions and other impermeable reagents.

X-ray diffraction and scattering also yield information concerning the number of lipid bilayers per phospholipid vesicle. As mentioned before, MLVs give rise to clearly defined diffraction (Bragg) maxima at low angles (cf. Chap. 11), while unilamellar vesicles are characterized by a diffuse scattering peak. Oligolamellar vesicles exhibit features in the diffraction pattern from which the number of lipid bilayers can be estimated, at least under appropriate conditions [76]. Even more direct and reliable, but tricky to perform, is the

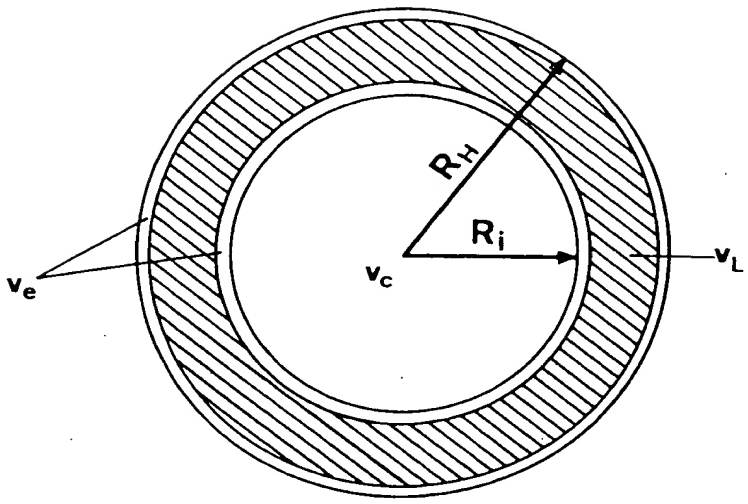

Figure 10 Scale diagram illustrating the various compartments of a phospholipid SUV; v_L is the volume of the anhydrous phospholipid bilayer, v_c is the internal volume, and v_e is the volume of water bound to the phospholipid polar groups.

lamellarity analysis by means of cryoelectron micrscopy using an energy filter for optimal contrast [77].

C. The Relationships Between Vesicle Dimensions Including Those of the Lipid Bilayer

The outer hydrodynamic or Stokes radius R_H of the hydrated vesicle (cf. Fig. 10) is obtained from various hydrodynamic measurements as discussed above. Alternatively, the outer radius R_H can be calculated from the specific volume $\bar{v}_h$ of the hydrated vesicle in mL/g of lipid, obtained from intrinsic viscosity measurements:

$$R_H = \left(\frac{3M_r\bar{v}_h}{4\pi N}\right)^{1/3} \tag{6}$$

$$[\eta] = \frac{v\bar{v}_h}{100} \tag{7}$$

where $[\eta]$ is the intrinsic viscosity of the vesicle suspension and v the Simha viscosity increment. The values of R_H obtained from the measurement of the diffusion coefficient D_0 according to Eq. (5) and from viscosity measurements according to Eqs. (6) and (7) are in good agreement as shown for SUVs of dimyristoyl phosphatidylcholine [78].

The inner radius R_i of the hydrated vesicle is obtained directly from the measurement of the trapped volume v_c (cf. Fig. 10)

$$R_i = \left(\frac{3Mv_c}{4\pi N}\right)^{1/3} \tag{8}$$

For the details of this measurement see Refs. 78 and 79. R_i can also be obtained from R_H if the thickness d_h of the hydrated phospholipid bilayer is known. A value for d_h can be derived experimentally from x-ray diffraction as discussed below.

$$R_i = R_H - d_h \tag{9}$$

The dimensions of the lipid bilayer can be calculated assuming a four-compartment model as depicted in Fig. 10. The specific volume of the hydrated phospholipid vesicle $\bar{v}_h$ can be expressed in this model as

$$\bar{v}_h = v_L + v_e + v_c \tag{10}$$

where v_L is the specific volume of the anhydrous bilayer, v_c is the trapped volume, and v_e is the additional volume of water bound to the outer and inner polar groups of the phospholipid bilayer. All volumes are specific volumes expressed as mL/g of phospholipid. The partial specific volume of the hydrated phospholipid $\bar{v}$ is then

$$\bar{v} = \bar{v}_h - \frac{w_t}{\rho} = v_L + v_c + v_e - \frac{w_t}{\rho} \tag{11}$$

where w_t is the total mass of water associated with the phospholipid in g water per g of lipid and ρ is the density of the suspending medium. The partial specific volume of the phospholipid vesicle can be also expressed in terms of the water of hydration

$$\bar{v} = v_L + \frac{w_b}{\rho'} - \frac{w_b}{\rho} \tag{12}$$

where w_b is the phospholipid hydration expressed as the mass of water bound to the phospholipid polar group in g water/g of phospholipid, and ρ' is the density of bound water. According to Eq. (12), the partial specific volume $\bar{v}$ is not identical to the specific volume v_L of the anhydrous phospholipid. However, if it is assumed to a first approximation that $\rho' \approx \rho$, then $\bar{v} = v_L$.

The specific volume of the hydrated phospholipid bilayer is $v_L + v_e = \bar{v} - v_c + w_t/\rho$; it can be obtained from the measurements of the outer (Stokes) radius R_H and the hydrated bilayer thickness d_h.

$$v_L + v_e = \frac{4\pi}{3}[R_H^3 - (R_H - d_h)^3] \tag{13}$$

For example, x-ray diffraction measurements performed with unsonicated egg phosphatidylcholine suspensions in water yield a d_h value of 4.56 nm [15]. From the knowledge of $v_L + v_e$ and that of the hydrated volume v_{PL} of one phospholipid molecule, a mean value for the total number n_t, of lipid molecules per vesicle is obtained:

$$n_t = \frac{v_L + v_e}{v_{PL}} \tag{14}$$

For example, the value for the hydrated volume of one egg phosphatidylcholine molecule was determined as $v_{PL} = 1.6368\ \text{nm}^3$ [15]. A mean value for n_t can also be calculated from the mean anhydrous vesicle weight M_r determined by analytical ultracentrifugation:

$$n_t = \frac{M_r}{M_L} \tag{15}$$

where M_L is the molecular weight of the phospholipid.

SUVs produced by exposing the phospholipid suspension to shear forces, such as by sonication and French press treatment, are believed to be under considerable stress due to the vesicle curvature. The relatively small radius of curvature of SUVs causes differences in the packing between the phospholipid polar groups on the outer and inner monolayer of the bilayer. This is manifested in the magnetic nonequivalence of the outer and inner polar groups of phospholipid SUVs observed by ^{1}H NMR. For instance, the polar group resonances of sonicated egg phosphatidylcholine vesicles exhibit a characteristic splitting [43,80–82] that was shown to increase with decreasing vesicle radius [82]. The tighter packing of the glycerophosphorylcholine group in the inner monolayer was suggested to lead to a partial dehydration and to conformational changes in the phospholipid polar group [82]. The coplanar orientation of the phospholipid group present in planar phosphatidylcholine bilayers [9] may change to a space-saving tilted orientation in the inner monolayer. Such a more perpendicular orientation of the inner polar groups gives rise to a net dipole moment along the bilayer normal and a net repulsion between phospholipid molecules in the inner monolayer, thus destabilizing the bilayer.

The splitting of the polar group signals observed in the ^{1}H NMR spectrum of SUVs of phosphatidylcholine can, in principle, be used to determine the molar ratio n_o/n_i where n_o and n_i are the number of phospholipid molecules located in the outer and inner monolayer of the vesicle bilayer, respectively. The splitting of the polar group signals can be artificially enhanced by the addition of paramagnetic shift reagents so that the resonances arising from the outer and inner monolayer are completely separated. In this case the molar ratio n_o/n_i is readily obtained from the integrated areas of the appropriate resonances [83,84]. Knowing the outer (R_H) and inner (R_i) radius of the hydrated phospholipid vesicle, the hydrated phospholipid areas in nm^2 per molecule can be calculated for the outer and inner vesicle surface using the relations

$$n_t = n_o + n_i \tag{16}$$

$$\frac{n_t}{n_i} = 1 + \frac{n_o}{n_i} \tag{17}$$

The hydrated phospholipid areas of the outer and inner vesicle surface, A_o and A_i, respectively, are then

$$A_o = \frac{4\pi R_H^2}{n_o} \tag{18}$$

$$A_i = \frac{4\pi R_i^2}{n_i} \tag{19}$$

D. Thermodynamic Stability of Phospholipid Vesicles

SUVs of phospholipids have been widely used as model membranes in the past, even though the precise relationship between the bilayers of SUVs and those of biological membranes is still unclear. In this context, the question of thermodynamic stability of SUVs is of prime importance. The information available on this question is scarce and often hidden in papers addressed to a different question.

To the best of my knowledge, no systematic study on the thermodynamic stability of phospholipid vesicles has been carried out so far. The information that is available to date

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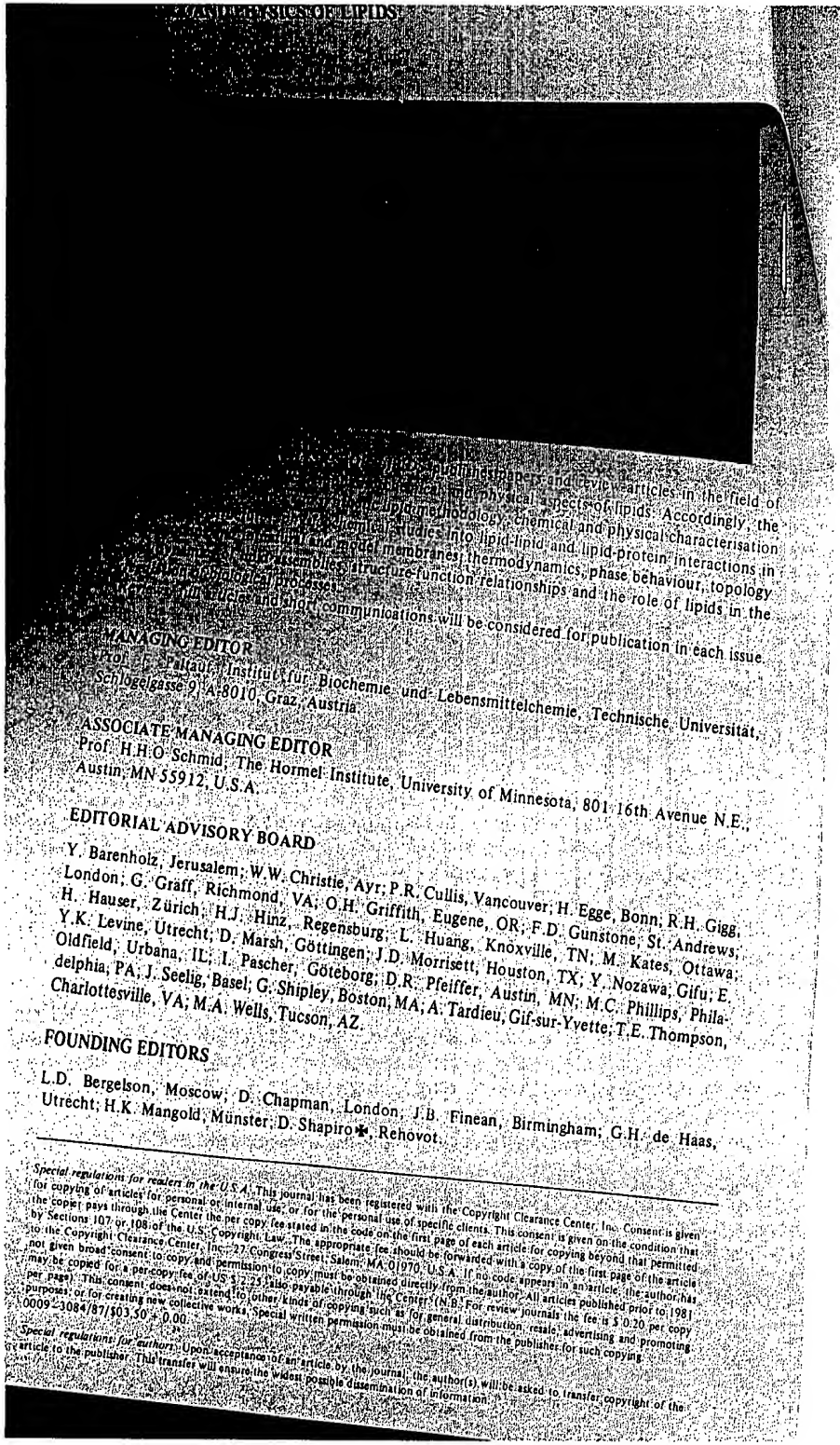

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PREDICTION OF THE CRITICAL MICELLE CONCENTRATIONS OF MONO- AND DI-ACYL PHOSPHOLIPIDS

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The experimental linear chainlength dependence of the logarithm of the critical micelle concentration (ln[CMC]) of mono- and di-acyl phosphatidylcholines, together with recent measurements of the polar headgroup dependence of the CMCs of a variety of spin-labelled phospholipids, and the relative solubilities of saturated and unsaturated hydrocarbons, have been combined to devise a scheme for making general predictions of the CMCs of both mono- and di-acyl phospholipids, such as are found in biological membranes.

Keywords: phospholipid; critical micelle concentration; micelle; bilayer.

Introduction

The thermodynamics of self assembly of membrane phospholipids is determined by the free energy of transfer, $\mu_{mic}^{\circ} - \mu_{w}^{\circ}$, of a lipid monomer from water to the micelle, which is characterized by the critical micelle concentration (CMC) (see e.g. Refs. 1 and 2). In addition, the CMC is frequently the controlling factor in the transfer of lipid molecules between membranes, in the cases for which this occurs via monomer diffusion (see e.g. Ref. 3). The CMC also contains some of the same energetic contributions as those affecting lipid membrane stability and cellular processes such as membrane fusion and biosynthesis [2,4]. Knowledge of the CMC is therefore important not only to a study of lipid transfer and membrane solubilization properties, but also to membrane assembly and stability in general.

In the present communication we try to present a general scheme for predicting the CMCs of phospholipids found in biological membranes, since most of these are not easily accessible experimentally. This is done by combining the available experimental information on the chainlength dependence of the CMCs of phosphatidylcholines, the polar headgroup dependence of the CMCs of spin labelled

Abbreviations: CMC, critical micelle concentration; PC, PA, PG, PS, PE, 1-acyl-2-acyl-*sn*-glycero-3-phosphocholine, -phosphoric acid, -phosphoglycerol, -phosphoserine, -phosphoethanolamine, respectively.

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transfer of saturated linear hydrocarbons from water to the hydrocarbon liquid (see Ref. 1):

$$\Delta G_{tr}/RT = -4.10 - 1.49\, n_{CH} \qquad (r^2 = 0.999) \tag{7}$$

For the *n*-alkenes, the corresponding expression is:

$$\Delta G_{tr}/RT = -2.54 - 1.49\, n_{CH} \qquad (r^2 = 0.997) \tag{8}$$

with little positional dependence between the 1- and 2-alkenes, and for the *n*-dialkenes is:

$$\Delta G_{tr}/RT = -1.51 - 1.46\, n_{CH} \qquad (r^2 = 0.999) \tag{9}$$

where the latter data is all for the 1-ω-dialkenes. Clearly the incremental free energies of transfer are very similar for both saturated and unsaturated chains, and therefore the effects of chain unsaturation on the CMCs can be approximated by contributions of 1.56RT and 2.59RT to the free energy of transfer for one and two double bonds, respectively. For the case of diacyl phospholipids with chain overlap, this contribution should be multiplied by 0.75, as discussed in the comparison of Eqns. (5) and (6).

The CMCs of a variety of phospholipids with different polar headgroups, but constant apolar part have been measured recently using spin label electron spin resonance [4]. This allows determination of the contributions of the different phospholipid polar headgroups to the free energy of transfer. The differences in the free energy of transfer ($\Delta G_{tr}^{pol}/RT$), normalized with respect to phosphatidylcholine in the absence of salt, are given for various salt concentrations in Table I. The salt dependence of the CMCs of the zwitterionic lipids, PC and PE, is much less than for the negatively charged lipids. The free energy of transfer for phosphatidylcholine has an approximately linear dependence on salt concentration given by:

$$\Delta G_{tr}^{pol}/RT = -0.672\ [NaCl] \qquad (r^2 = 0.987) \tag{10}$$

As an approximate alternative to using the experimental values, the salt dependence for the negatively charged lipids can be estimated from the Debye-Hückel expression for the ionic strength dependence of the electrostatic free energy of the micelle [1]:

$$\Delta G_{el} = [N_A z^2 e^2 \bar{n}/2\epsilon]\ [\kappa/(1 + \kappa a)] \tag{11}$$

where ϵ is the effective dielectric constant at the micelle-water interface, a is the encounter distance between centres of micelle and counter-ion, $\bar{n}$ is the average micellar number, z is the charge on the lipid monomer, e is the electronic charge, k is Boltzmann's constant, and $\kappa = (8\pi N_A e^2 I/1000\epsilon kT)^{1/2}$ is the reciprocal Debye-Hückel screening length in a 1:1 electrolyte of ionic strength I, N_A being Avogadro's number. It was shown in Ref. 4 that the data in Table I can be fitted approximately by this expression for the singly negatively charged lipids over the range 0.01–1.0 M NaCl, with parameters of the following values: $\epsilon = 80$ and $a = 31$ Å and $\bar{n} = 63$. The electrostatic contribution given in Eqn. (11) is additive to the free energy of transfer in Eqn. (3), and hence its effect on the CMC can be calculated.

The CMCs of phospholipids with different headgroups and hydrocarbon chain compositions can now be estimated by combination of Eqns. (5) to (9) with Table I. For monoacyl phospholipids, the CMC in mole fraction units is approximated by:

$$\ln[CMC] = \Delta G_{tr}^{pol}/RT + 0.8 - 1.1\, n_{CH} + 1.6 n_{unsat} - 0.5(n_{unsat} - 1) \tag{12}$$

where $\Delta G_{tr}^{pol}/RT$ is the polar group contribution relative to phosphatidylcholine from Table I, and n_{unsat} is the number of double bonds in the chain. The final term on the right is the correction for the decreased effect of double bonds beyond the first (cf. Eqns. 8 and 9), and is only applicable for $n_{unsat} \geq 1$. The corresponding expression for diacyl phospholipids with chains of equal length is:

$$\ln[CMC] = \Delta G_{tr}^{pol}/RT - 0.4 - 1.7\, n_{CH} + 1.2\, n_{unsat} - 0.4(n_{unsat} - 1) \tag{13}$$

where n_{CH} is the number of aliphatic C-atoms in one chain, and n_{unsat} is the total number of double bonds in the two chains. For mixed-chain diacyl lipids, the dependence of the CMC on chainlength and degree of unsaturation is given by

TABLE I

FREE ENERGIES OF MONOMER-MICELLE TRANSFER ($\Delta G_{tr}^{pol}/RT$) RELATIVE TO PC (IN THE ABSENCE OF SALT) FOR DIFFERENT *sn*-2 SPIN-LABELLED PHOSPHOLIPIDS WITH FIXED CHAIN COMPOSITION IN NaCl AT 20°C AND pH 7, EXCEPT FOR PA^{2-} AND PAH^- WHICH REPRESENT PHOSPHATIDIC ACID AT pH 8 AND pH 5, RESPECTIVELY

From Ref. 4.

[NaCl] (M)	PA^{2-}	PAH^-	PS^-	PG^-	PC	PE
0.0	–	–	+2.7	+2.2	0.0	−0.7
0.15	+1.9	+0.1	+0.7	+0.4	−0.2	−0.9
0.5	+0.7	−0.5	−0.2	−0.3	−0.3	−0.9
1.0	+0.1	−1.1	−0.9	−0.8	−0.8	−1.1
2.0	−1.0	−2.0	−1.7	−1.8	−1.3	−2.0

Phospholipids Handbook

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I. CRITICAL CONCENTRATION FOR THE SELF-AGGREGATION OF VARIOUS PHOSPHOLIPIDS AS A FUNCTION OF POLAR HEAD GROUP AND HYDROCARBON CHAIN LENGTH

$$\ln[\mathrm{CMC}] = \Delta G_{tr}^{pol}/RT - 0.4 - 1.7\, n_{CH}^{s} - 1.1(n_{CH}^{l} - n_{CH}^{s}) + 1.2\, n_{unsat}^{0} - 0.4(n_{unsat}^{0} - 1) + 1.6\, n_{unsat}^{n} - 0.5(n_{unsat}^{n} - 1) \quad (1)$$

where n_{CH}^{l} and n_{CH}^{s} are the numbers of aliphatic C-atoms in the longer and shorter chains, respectively, and n_{unsat}^{0} and n_{unsat}^{n} are the numbers of double bonds in the overlapping and nonoverlapping regions of the chains, respectivley. Conversion from mole fraction units to molar concentrations can be made by adding 4.0 to the right-hand side of Eq. 1

Table C2 Free Energies of Monomer-Micelle Transfer ($\Delta G_{tr}^{pol}/RT$) Relative to PC (in the Absence of Salt) for Different *sn*-2 Spin Labelled Phospholipids with Fixed Chain Composition in NaCl at 20°C and pH 7, Except for PA^{2-} and PAH^{-}, Which Represent Phosphatidic Acid at pH 8 and pH 5, Respectively

[NaCl] (M)	PA^{2-}	PA^{-}	PS^{-}	PG^{-}	PC	PE
0.0	—	—	+2.7	+2.2	0.0	–0.7
0.15	+1.9	+0.1	+.07	+0.4	–0.2	–0.9
0.5	+0.7	–0.5	–0.2	–0.3	–0.3	–0.9
1.0	+0.1	–1.1	–0.9	–0.8	–0.8	–1.1
2.0	–1.0	–2.0	–1.7	–1.8	–1.3	–2.0

Table C3 Experimentally Determined CMCs Compared with the Predictions from Eq. (1), for 1-Acyl-2-Acetyl- and 1-Palmitoyl-2-Acyl-*sn*-Glycero-3-Phosphocholines

Chains	[CMC] (mol liter^{-1})	
	Expt.	Eqn. (1)
1-Acyl-2-acetyl		
1-(12:0)-2-(2:0)	$(1.1 \pm 0.25) \cdot 10^{-4}$	$1.1 \cdot 10^{-4}$
1-(14:0)-2-(2:0)	$(1.1 \pm 0.12) \cdot 10^{-5}$	$1.2 \cdot 10^{-5}$
1-(16:0)-2-(2:0)	$(1.3 \pm 0.06) \cdot 10^{-6}$	$1.4 \cdot 10^{-6}$
1-(18:0)-2-(2:0)	$(2.2 \pm 0.1) \cdot 10^{-7}$	$1.5 \cdot 10^{-7}$
1-Palmitoyl-2-acyl		
1-(16:0)-2-(2:0)	$(1.3 \pm 0.06) \cdot 10^{-6}$	$1.4 \cdot 10^{-6}$
1-(16:0)-2-(3:0)	$(7.2 \pm 0.3) \cdot 10^{-7}$	$7.5 \cdot 10^{-7}$
1-(16:0)-2-(4:0)	$(4.1 \pm 0.2) \cdot 10^{-7}$	$4.1 \cdot 10^{-7}$
1-(16:0)-2-(6:0)	$(2.2 \pm 0.1) \cdot 10^{-7}$	$1.2 \cdot 10^{-7}$

CMCs determined from W. Kramp, G. Pieroni, R. N. Pinckard, and D. J. Hanahan, *Chem. Phys. Lipids* 35:49–62 (1984).
Source: D. Marsh and M. C. King. *Chem. Phys. Lipids* 42:271–277 (1986).

Lipid Membrane Partitioning of Lysolipids and Fatty Acids: Effects of Membrane Phase Structure and Detergent Chain Length§

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The lipid membrane partitioning of lysolipids (lysoPC) and fatty acids (FA) into unilamellar vesicles composed of saturated $DC_{16}PC$ phospholipids has been determined by means of isothermal titration calorimetry (ITC). The calorimetric titrations were performed at low temperatures in the ordered gel phase and at high temperatures in the disordered fluid phase of the phospholipid membrane vesicles. The long saturated acyl chains of the lysolipids and fatty acids varied from 10 to 16 carbon atoms and all titrations were performed below the critical micellar concentrations (cmc) of the detergents. The calorimetric results reveal that the membrane partitioning of lysolipids depends strongly on the phase structure of the lipid membrane. This is manifested as a lysolipid partition coefficient, K, that is much larger for fluid-phase lipid membranes as compared to gel-phase lipid membranes. Oppositely, the membrane partitioning of fatty acids depends only weakly on the phase structure of the phospholipid vesicles. In addition, the thermodynamic measurements show that the partition coefficients for both the lysolipids and fatty acids toward gel and fluid lipid membranes become almost an order of magnitude higher when the saturated acyl chain of the detergents increases by two carbon atoms. The obtained partition coefficients are of importance in relation to a deeper understanding of the interplay between global aqueous and local membrane concentrations of the detergents and the functional influence on, for example, the lipid membrane permeability and the activity of membrane associated enzymes such as phospholipase A_2.

Introduction

The lipid bilayer part of a biological membrane is built of a large number of different lipid species which form the physical boundary that separates and encapsulates the cytoplasm and the cell organelles.[1−3] The variety of lipids constituting the lipid bilayer as well as water-soluble amphipilic agents, e.g., free fatty acids and lysolipids that distribute themselves between the aqueous phase and the lipid bilayer phase, are major determinants of the macroscopic phase behavior of the lipid bilayer system.[4,5] The transfer of water-soluble detergents from the aqueous phase to the lipid membrane is described by an equilibrium partition coefficient and it is well-established that above a certain detergent concentration, that usually is given in terms of a global concentration of exogeneously added detergents, the lipid membrane breaks down and forms mixed micellar structures.[4,5] However, it is the local concentration of the lysolipids and/or the fatty acids in the lipid membrane that is of relevance for the membrane perturbing effects induced by the detergents. Therefore, a detailed knowledge of the local concentration of the detergents in the membrane is required.[6−8] Furthermore, both the equilibrium and nonequilibrium local lateral distribution of the detergents in the heterogeneous membrane structure might be of relevance for membrane function.[7,9,10−13]

The partitioning of water-soluble amphiphiles such as free fatty acids and lysolipids between the aqueous phase and the lipid bilayer phase is of importance not only from a pure physicochemical point of view, but also in relation to a large number of biological and biotechnological processes where the amphiphilic agents act as regulatory lipids for the functioning of several lipid membrane associated processes. The incorporation of nonbilayer-forming agents into the membrane leads to a change in the propensity for forming a hexagonal phase which is of importance for biomaterial properties such as lipid membrane stability and bending rigidity[12] as well as for the functioning of membrane-associated enzymes, peptides, and proteins.[14] Important examples include the fusogenic behavior of vesicles,[15] the functioning of lipid membrane associated enzymes such as phospholipase A_2 (PLA_2),[16] the ability of antibacterial gramicidin monomer peptides to form ion-conducting dimer channels,[17,18] and the activity of integral channel-forming proteins.[19] Furthermore, important drug and drug-delivery aspects are closely related to the way amphiphilic solutes such as lysolipids and free fatty acids incorporate into lipid membranes[12,20,21] and distribute themselves laterally in the heterogeneous membrane structure.[8,9,22]

The intensively studied phospholipase A_2-catalyzed degradation of lipid membranes, which leads to the formation of lysolipid and free fatty acid hydrolysis products, is a particular prominent example of the close relationship that exists between lipid membrane-associated enzymatic activity and the presence of detergents in the lipid membrane.[23−29] Not only has it been demonstrated that the incorporation of a critical concentration of the products in the lipid membrane leads to spontaneous activation of the enzyme, but it has also been argued that the sudden change in lipid membrane composition that takes place due to the fast generation of PLA_2-catalyzed hydrolysis products, leads to long-living nonequilibrium small-scale phase separated structures[16] similar to the nonequilibrium lipid domains that have been observed in two-component lipid mixtures after a sudden temperature quench.[10] Furthermore, it is known that the PLA_2-generated hydrolysis products have a significant influence on the mechanical properties of the lipid bilayer, e.g., the bilayer bending rigidity[30] and the transmembrane barrier properties of well-defined vesicle membranes[31] as well as on lysis events of the lipid membrane part of red blood cells.[32] In particular, it has been observed that the lysis capability and the permeability-enhancing effect of the detergents are strongly determined by the phase structure of the lipid membrane.[32] This is manifested as a minor perturbing and permeability-enhancing effect toward fluid lipid membranes, whereas a dramatic enhancement of the permeability and lysis events are observed when the lipid membrane is in the ordered gel state.[32] Whether these observations reflect different molecular mechanisms involved in the membrane perturbing effects of disordered and ordered lipid membranes, or they simply reflect a different partitioning and local concentration of the detergents in the lipid membrane is unclear.

However, a prerequisite for an increased understanding of the molecular mechanisms involved in the functional membrane perturbing effects of lysolipids and free fatty acids requires a detailed knowledge of the actual local concentration of the detergents in the membrane and in particular how the membrane partitioning depends on the phospholipid membrane phase structure as well as on the chain length of the amphiphilic agents.[29,33,34] A limited number of studies involving fluorescence[35,36] have been directed toward a systematic chain-length-dependent characterization of the lipid membrane partitioning of amphiphilic detergents such as free fatty acids and lysolipids.

In this study, we have by means of isothermal titration calorimetry (ITC)[37,38] undertaken a systematic investigation of the lipid membrane partitioning of different fatty acids and lysolipids into unilamellar vesicles composed of $DC_{16}PC$ (DPPC) lipids. Advantageously, this method does not require the usage of lipid membrane perturbing probes in contrast to electron spin resonance and fluorescence techniques. The lipid membrane partition coefficients are determined at temperatures above and below the gel-to-fluid phase transition temperature, T_m, of the $DC_{16}PC$ vesicles, e.g., in the ordered gel phase and the disordered fluid phase of the lipid membrane. The chain lengths of the free fatty acids and lysolipids varied from 10 to 16 carbons in the long saturated acyl chains. All titrations were performed below the critical micellar and the bilayer-to-micellar concentrations of the detergents.

Materials and Methods

Materials. The lipids 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine ($DC_{16}PC$), 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine ($lyso_{16}PC$), 1-myristoyl-2-hydroxy-*sn*-glycero-3-phosphocholine ($lyso_{14}PC$), 1-lauroyl-2-hydroxy-*sn*-glycero-3-phosphocholine ($lyso_{12}PC$), 1-capryl-2-hydroxy-*sn*-glycero-3-phosphocholine ($lyso_{10}PC$) were purchased from Avanti Polar Lipids (Alabaster, AL). Palmitic acid ($F_{16}A$), myristic acid ($F_{14}A$) were from Avanti Polar Lipids, and lauric acid ($F_{12}A$) and capric acid ($F_{10}A$) were from Sigma (St. Louis, MO). All chemicals were used without further purification. Saturated lysophosphatidylcholine with n carbon atoms in the saturated acyl chain ($lyso_nPC$) and sodium salts of the free fatty acid with n carbon atoms in the acyl chain (F_nA) were weighed and dissolved in Milli-Q water (18 MΩ) containing 10 mM HEPES and 10 μM EDTA (pH 7.5). The $F_{16}A$ and $F_{14}A$ aqueous mixtures were heated to 65 °C before each experiment in order to ensure complete dissolution of the fatty acids prior to titration.

Preparation of Large Unilamellar Vesicles (LUV). Multilamellar vesicles were made by dispersing a weighed amount of $DC_{16}PC$ lipids in the aqueous buffer. The lipid suspension was kept at a temperature 10 °C above the main phase transition of $DC_{16}PC$ (T_m = 41.5 °C) for 1 h in order to ensure complete hydration. During this period the lipid suspension was vortexed every 15 min. The multilamellar vesicles were extruded 10 times through two stacked 100 nm pore size polycarbonate filters forming unilamellar vesicles (LUV) with a narrow size distribution.[39] The lipid concentration of the vesicles was determined by HPLC before and after extrusion and it was found that less than 5% of the $DC_{16}PC$ lipid material was lost during the extrusion procedure.

Isothermal Titration Calorimetry. Isothermal titration calorimetry was performed using a VP isothermal titration calorimeter from Microcal (Northampton, MA). The $DC_{16}PC$ lipid vesicle suspension and the F_nA and $lyso_nPC$ aqueous solutions were degassed under low pressure before use to avoid the formation of air bubbles during the titrations. Each experiment consists of a series of injections of the $DC_{16}PC$ vesicle suspension from a 300 μL syringe into the 1.40 mL titration cell loaded with aqueous solutions of F_nA or $lyso_nPC$. The differential power needed to maintain zero temperature difference between the reference cell and the sample cell after injection of vesicle titrant is recorded as a function of time. The experimental data were evaluated using the MicroCal Observer and Origin software and converted to heat of lipid membrane partitioning, h_i, of the detergents after each injection step, i. The actual concentrations of the lipid components in the cell are calculated and correcting for the small dilution effects due to injection of the vesicle suspension.

Partitioning Model. Lipid vesicles were injected into aqueous solutions of fatty acid or lysolipid and the measured heat of transfer, h_i, after each injection, i, was used on basis of an equilibrium partitioning model to determine the partition coefficient, K, and the molar enthalpy of partitioning, ΔH, of the F_nA and $lyso_nPC$ detergents between the aqueous phase and the lipid membrane phase.[4] Only concentrations below the critical micellar and the bilayer-to-micellar concentrations of the detergents are used in the titrations ensuring that lipid membranes of variable composition are present during each titration experiment. This approach implies that the lipid membrane acts as a solvent for the detergents and that the partition coefficient is concentration-independent. The equilibrium partitioning of the detergents between the aqueous phase and the lipid vesicles can then be described as a simple partitioning of the detergents between two thermodynamic bulk phases:

$$D_w \rightleftharpoons D_m \qquad (1)$$

where D_w and D_m refers to free aqueous and lipid membrane states of the detergent, respectively. From the equilibrium

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§ Abbreviations used: ITC: isothermal titration calorimetry; DC_nPC: saturated di-acyl phosphatidylcholine with n carbon atoms in each saturated acyl chain; DPPC: 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; $lyso_nPC$: saturated lyso-phosphatidylcholine with n carbon atoms in the saturated acyl chain; F_nA: free fatty acid with n carbon atoms in the saturated acyl chain; cmc: critical micellar concentration; K: partition coefficient.

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reaction in eq 1 the partition coefficient can be defined as

$$K_w = \frac{C_{D_m}}{C_L}\frac{C_w}{C_{D_w}} = C_w K \quad (2)$$

where $C_w = 55$ M is the molar concentration of water, and C_{D_m} and C_{D_w} are the equilibrium concentration of the detergent in the lipid membrane phase and the aqueous phase, respectively. C_L refers to the total lipid concentration. The concentration of detergent in the lipid membrane can then be rewritten as

$$C_{D_m} = \frac{KC_L C_D}{KC_L + 1} \quad (3)$$

where C_D is the total detergent concentration in the titration chamber. The concentration of lipid, C_L, in the titration chamber of volume, V^0, is given by

$$C_L = \frac{C_L^0 iV_{inj}}{V^0 + iV_{inj}} \quad (4)$$

where C_L^0 refers to the concentration of lipid in the titration syringe and i and V_{inj} are the titration injection number and the injection volume, respectively. The overall concentration of detergent in the chamber, C_D, is given by

$$C_D = \frac{C_D^0 V^0}{V^0 + iV_{inj}} \quad (5)$$

where C_D^0 is the initial concentration of detergent in the titration chamber.

It is assumed that the accumulated heat of partitioning, H_i, is proportional to the amount of detergent that has partitioned into the lipid membrane:

$$H_i = \Delta H n_{D_m} = \Delta H C_{D_m}(V^0 + iV_{inj}) \quad (6)$$

where ΔH is the molar enthalpy of partitioning and n_{D_m} is the molar amount of detergents bound to the lipid membrane.

The accumulated heat can be written in terms of the initial concentrations:

$$H_i = \Delta H C_L^0 C_D^0 V^0 \frac{Ki}{KC_L^0 i + \frac{V^0}{V_{inj}} + i} \quad (7)$$

From the titration experiment we get the heat of the consecutive injections, h_i:

$$h_i = H_i - H_{i-1} \quad (8)$$

The calculation takes into account the small and presumably constant heat of dilution of the injection of $DC_{16}PC$ vesicles into aqueous buffer in the titration chamber.[40] By least-squares analysis, the partition coefficient, K, and the molar enthalpy, ΔH, of the equilibrium reaction in eq 1 can be calculated. Examples of the best fit to the calorimetric data for titrations of $DC_{16}PC$ vesicles into F_nA and $lyso_nPC$ solutions can be seen in Figures 1 and 2.

Results

All the titration experiments of the $DC_{16}PC$ vesicles into the aqueous mixtures of fatty acids and lysolipids were performed below the critical micellar concentrations of the detergents. Only a limited number of critical micellar concentration (cmc) values for the detergents used in the present study have been reported earlier as shown in Table 1. By means of a simple isothermal titration method,[42] we have prior to the partitioning experiments determined cmc values of the detergents. Briefly, the method involves consecutive injections of a micellar detergent suspension into aqueous buffer. After the first injections the micelles are completely dissolved, resulting in a large demicellization titration heat. When the detergent concentration in the cell increases no further demicellization takes place; from the vanishing heat evolved, the cmc value of the detergent can be determined as the inflection point of the resulting titration curve.[42] Table 1 contains critical micelle concentrations for all the F_nA and $lyso_nPC$[41] detergents used in the present study. The established cmc values for F_nA are obtained as described above. The cmc values for both fatty acids and lysolipids increase with chain length ranging from 1.3×10^{-6} to 6×10^{-3} M. The results listed in Table 1 enable us to perform partition coefficient experiments in the desired concentration range, e.g., using aqueous monomeric solutions of the lysolipid and fatty acid detergents in the titration cell.

Figures 1A and 1B show the calorimetric data from the isothermal titrations obtained at 30 °C of a $DC_{16}PC$ unilamellar vesicle suspension titrated into aqueous fatty acid solutions of $F_{12}A$ (Figure 1A) and $F_{14}A$ (Figure 1B). Each injection of the $DC_{16}PC$ unilamellar vesicle suspension produces an exothermic heat of reaction, which decreases with injection number as less and less free fatty acids are present in the aqueous phase. In Figures 1C and 1D (lower panel) the integrated titration peaks corresponding to the heat of transfer, h_i, of fatty acids from the aqueous phase into the $DC_{16}PC$ gel phase membrane, are shown as a function of the lipid concentration, C_L, in the titration cell, e.g., the number of injections, i. It should be remarked that only a small heat of dilution was measured when the vesicle suspension in separate experiments was titrated into pure buffer (data not shown). The solid lines in Figures 1C and 1D represent the best fits obtained by a nonlinear least-squares optimization routine of the measured titration data to the equilibrium partitioning model described by eqs 7−8. The fits in Figures 1C and 1D for $F_{12}A$ and $F_{14}A$, which are based on the simple two-state thermodynamic partitioning model described by eq 1, are in good accordance with the experimentally obtained data. From Figures 1 and 2 it is noticed that the transfer of the $F_{12}A$ and $F_{14}A$ fatty acids from the aqueous phase into the membrane phase involves a large exothermic enthalpy of partitioning. The chain-length-dependent partitioning coefficients, K, and the enthalpies of partitioning, ΔH, for $F_{10}A$, $F_{12}A$, $F_{14}A$, and $F_{16}A$ fatty acids into gel and fluid phase $DC_{16}PC$ vesicles are listed in Table 2. Only the partitioning of $F_{10}A$, into gel phase DC_{16}-PC vesicles involves an endothermic enthalpy of partitioning, ΔH, as shown in Table 2. From Table 2 it is furthermore seen that the partition coefficient, K, increases by nearly an order of magnitude, when the long hydrophobic acyl chain of the fatty acids increases by two methylene units. Interestingly, Table 2 also reveals that the partition coefficients for the fatty acids investigated are only weakly influenced by the underlying gel- or fluid-phase structure of the $DC_{16}PC$ vesicles characterized by a gel-to-fluid phase transition at $T_m = 41.5$ °C.[8] We shall below return to a more detailed discussion of these effects.

The upper panels in Figure 2 display the experimental data obtained from titrations of a suspension of $DC_{16}PC$ LUV into solutions of $lyso_{12}PC$ and $lyso_{14}PC$. In the lower panels the integrated peaks are shown together with least-squares fits of

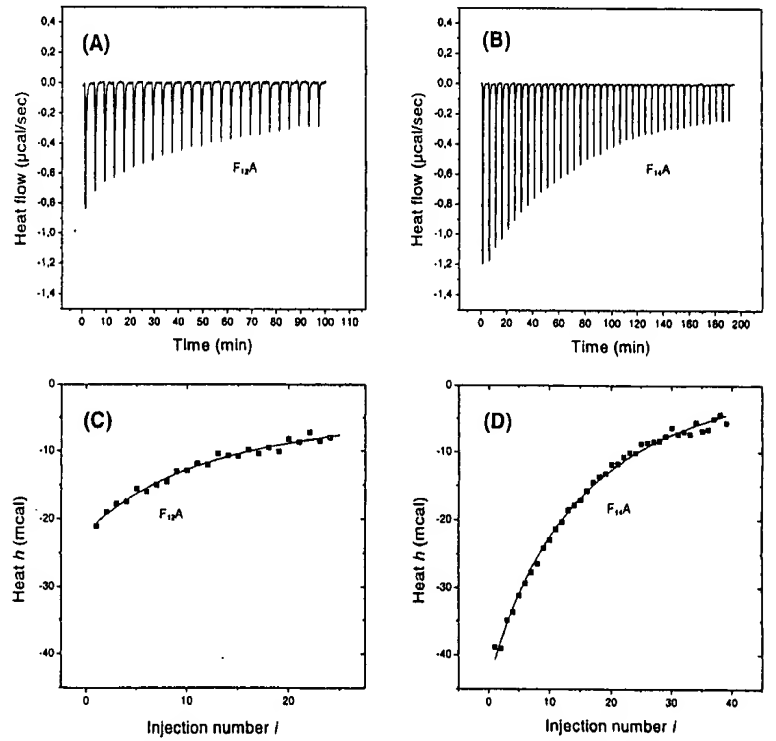

Figure 1. Isothermal titration calorimetry of free fatty acid solutions with unilamellar $DC_{16}PC$ vesicles at 30 °C (upper panels) and the heats of transfer, h_i, of the detergents into the lipid membrane phase (lower panels) as obtained from integration of the titration peaks. (A) Each peak corresponds to the injection of 5 µL of 20 mM $DC_{16}PC$ vesicle suspension into 200 µM of a $F_{12}A$ solution. (B) Each peak corresponds to the injection of 8 µL of 1 mM $DC_{16}PC$ vesicle suspension into 100 µM of a $F_{14}A$ solution. The solid lines represent least-squares fits of the calorimetric data to the equilibrium partioning model described in eqs 7−8 using $K = 470$ M^{-1} and $\Delta H = -2170$ cal/mol for $F_{12}A$ (C), and $K = 5000$ M^{-1} and $\Delta H = -12500$ cal/mol for $F_{14}A$ (D).

the partitioning model to the integrated calorimetric data as described in eqs 1−8. The calculated partition coefficients, K, and molar enthalpies of partitioning, ΔH, for all the lysolipids investigated are listed in Table 3. The molar enthalpies in the case of $lyso_nPC$ in general are much lower than the values shown in Table 2 for F_nA. The low cmc values of in particular the long chain lysolipids (see Table 1) limit the total amount of those long chain lysolipids in the titration cell. In addition, the high partition coefficients of the lysolipids listed in Table 3 indicate that only small amounts of $DC_{16}PC$ is needed to absorb most of the free $lyso_nPC$ from the aqueous phase and solubilize the vesicles (induce a bilayer-to-micellar transition) when the vesicles are titrated into the sample cell. Thus, only low concentrations of $DC_{16}PC$ and lysolipid detergents can be used in the titrations, and as a consequence only a low heat of partitioning is detectable after each injection. These experimental constraints lead to a high degree of uncertainty in the calculated values of the partioning coefficient as seen in Table 3. By comparison of the values for the molar enthalpies of partitioning, ΔH, listed in Tables 2 and 3, it is apparent that the enthalpies of partitioning are significantly larger for F_nA than for the corresponding $lyso_nPC$.

Figure 3 displays the partitioning coefficients of $lyso_nPC$ (upper panel) and F_nA (lower panel) into $DC_{16}PC$ LUV as a function of the detergent acyl chain length. In the semilogarithmic plot in Figure 3 it is clearly seen that the partition coefficient, K, increases with the acyl chain length of the detergent for all the detergents studied. This effect is most pronounced for the fatty acids where the partition coefficient increases by almost a factor of 12 for every two methylene groups the acyl chain length increases. In the case of the lysolipids, the partition coefficient increases by approximately a factor of 6. Furthermore, it is seen that the partition coefficients for the fatty acids are almost independent of the phase structure of the $DC_{16}PC$ vesicles, e.g., ordered gel membrane structure at low temperatures (20 and 30 °C) or disordered fluid membrane structure at high temperatures (50 and 60 °C). For the lysolipids it is apparent that the underlying gel- or fluid-phase structure of the vesicles plays an important role for the partitioning of the lysolipid detergents into the lipid membrane.

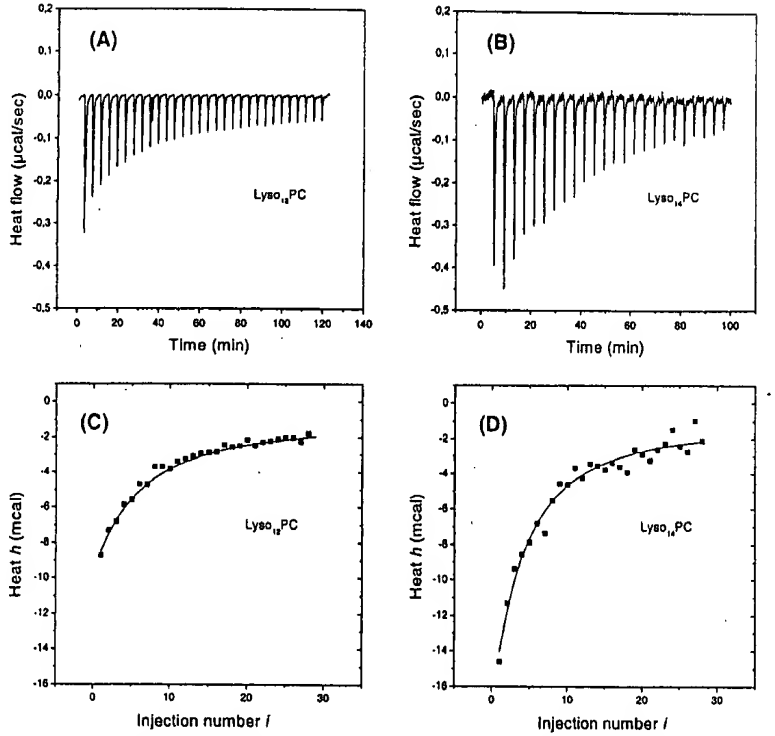

Figure 2. 2. Isothermal titration calorimetry of lysophospholipid solutions with unilamellar $DC_{16}PC$ vesicles at 30 °C (upper panels) and the heats of transfer, h, of the detergents into the lipid membrane phase (lower panels) as obtained from integration of the titration peaks. (A) Each peak corresponds to the injection of 5 μL of 20 mM $DC_{16}PC$ vesicle suspension into 200 μM of a $lyso_{12}PC$ solution. (B) Each peak corresponds to the injection of 3 μL of 10 mM $DC_{16}PC$ vesicle suspension into 50 μM of $lyso_{14}PC$ solution. The solid lines represent least-squares fits of the calorimetric data to the equilibrium partitioning model described in eqs 7–8 using $K = 1200\ M^{-1}$ and $\Delta H = -50$ cal/mol for $lyso_{12}PC$ (C), and $K = 8000\ M^{-1}$ and $\Delta H = -572$ cal/mol for $lyso_{14}PC$ (D).

This is clearly manifested as a significantly higher partitioning of lysolipids into disordered fluid-phase membrane than into ordered gel-phase membranes. The temperature dependence of the partition coefficient is displayed in Figure 4 for lysolipids (upper panel) and fatty acids (lower panel). For all the $lyso_nPC$ investigated the partioning coefficient increases with increasing temperature, while for the F_nA there seems to be no general effect of temperature on the partitioning coefficient.

TABLE 1: Critical Micellar Concentrations (cmc) for Fatty Acids, F_nA, and Lysolipids, $Lyso_nPC$, at 20 and 60 °C

	$cmc_{20°C}$ (M)	$cmc_{60°C}$ (M)
$F_{10}A$	5×10^{-3}	1.6×10^{-2}
$F_{12}A$	8×10^{-4}	1.4×10^{-3}
$F_{14}A$	2×10^{-4}	3×10^{-4}
$F_{16}A$	1.3×10^{-6}	2×10^{-5}
$lyso_{10}PC$	6×10^{-3}[a]	9.5×10^{-3}
$lyso_{12}PC$	4.3×10^{-4}[a]	0.6×10^{-3}
$lyso_{14}PC$	3.8×10^{-5} 4.3×10^{-5}[a]	5.5×10^{-5}
$lyso_{16}PC$	3.1×10^{-6} 4.0×10^{-6}[a]	6.5×10^{-6}

[a] Marsh, 1990.

Discussion and Conclusion

The transfer of fatty acids and lysolipids from the aqueous phase to the lipid membrane phase has been investigated using high-sensitivity isothermal titration calorimetry. By means of a simple thermodynamic partitioning model and the measured titration energies involved in the transfer of the detergents from the aqueous phase to the lipid membrane phase, the chain-length-dependent partition coefficients of various detergents were established into ordered and disordered bulk lipid membrane phases. The acyl chain lengths of the fatty acids and lysolipids varied from 10 to 16 carbon atoms, whereas unilamellar vesicles composed of saturated $DC_{16}PC$ phospholipids were used as the lipid membrane phase. The titrations were performed below and above the $DC_{16}PC$ gel-to-fluid phase transition temperature, T_m = 41.5 °C, in the ordered gel phase and at high temperatures in the disordered fluid phase. All the titration experiments were performed below the critical micellar concentration (cmc) of the detergents, suggesting that the detergents distribute themselves beween two bulk phases—the aqueous phase and the lipid membrane phase. In the model description it is assumed that

TABLE 2: Partition Coefficients, K, and Molar Enthalpies of Partitioning, ΔH, of Fatty Acids, F_nA, into Unilamellar $DC_{16}PC$ Vesicles at 20, 30, 50, and 60 °C Calculated According to the Thermodynamic Partitioning Model Described in Eqs 7,8

	temperature (°C)	partition coefficient (M^{-1})	ΔH (cal/mol)
$F_{10}A$	20	65	2100
	30	47	1300
	50	52	−4100
	60	49	−4100
$F_{12}A$	20	740	−2700
	30	470	−2200
	50	530	−2900
	60	750	−3000
$F_{14}A$	20	7000	−7200
	30	5000	−12500
	50	7900	−6800
	60	13400	−1100
$F_{16}A$	20	103000	−2900
	30	75000	−2900
	50	52500	−1200
	60	161000	−1800

TABLE 3: Partition Coefficient, K, and Molar Enthalpy of Partitioning, ΔH, of Lysolipids, $lyso_nPC$, into Unilamellar $DC_{16}PC$ Vesicles at 20, 30, 50, and 60 °C Calculated According to the Thermodynamic Partitioning Model Described in Eqs 7,8

	temperature (°C)	partition coefficient (M^{-1})	ΔH (cal/mol)
$lyso_{10}PC$	20	170	−52
	30	780	−36
	50	1700	−95
	60	1750	−26
$lyso_{12}PC$	20	1350	−230
	30	1200	−50
	50	4850	−230
	60	4100	−580
$lyso_{14}PC$	20	2200	−30
	30	8000	−570
	50	12000	−190
	60	21900	−1900
$lyso_{16}PC$	20	14100	−680
	30	11300	−940
	50	93000	91
	60	109000	38

the lipid membrane phase act as a homogeneous thermodynamic one-phase system; e.g., no phase separation takes place within the two-dimensional lipid membrane structure, implying that a linear relation exists between the molar amount of detergents, n_{D_m}, that has partitioned into the lipid membrane vesicles and the accumulated heat of partitioning, H_i, after i titrations. It should be mentioned, that when the titrations were performed at temperatures in the neighborhood of the main phase transition temperature, T_m, of the $DC_{16}PC$ vesicles, a nonlinear dependence on the amount of detergents partitioned into the lipid membrane and the heat of partioning was observed (data not shown). This observation implies a concentration-dependent influence of the detergents on the physical lipid membrane in terms of, for example, two-dimensional phase separation and/or a change in the main phase transition temperature, T_m, of the $DC_{16}PC$ lipid membrane. By means of a simple equilibrium thermodynamic model that assumes a partitioning equilibrium of the detergents between the bulk aqueous phase and the bulk gel or fluid lipid membrane phases (10 °C above and below T_m, respectively), the partition coeffecients, K, for the various detergents were calculated on the basis of the measured heat of

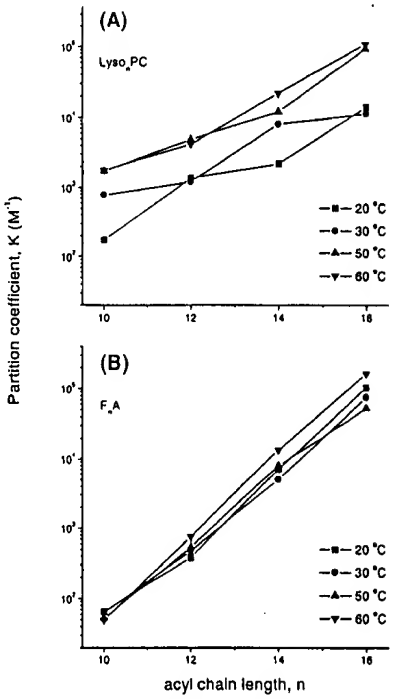

Figure 3. Semilogarithmic plot of the partition coefficient, K, for lysolipids and fatty acids into $DC_{16}PC$ vesicle as a function of acyl chain length, n, of the detergents. (A) The variation of the partition coefficient, K, for $lyso_nPC$ at 20, 30, 50, and 60 °C. (B) The variation of the partition coefficient, K, for F_nA at 20, 30, 50, and 60 °C.

partitioning. For most of the lysolipid and fatty acid detergents studied, the transfer from the aqueous phase to the membrane phase is found to be exothermic.

The results in Figures 1 and 2 provide a comparison of the experimentally obtained titration energies and the thermodynamic partitioning model. The smooth curves were calculated on basis of eqs 7–8, and it is evident that the simple thermodynamic partitioning model gives an excellent description of the high sensitivity calorimetric partitioning results of the detergents between the two bulk phases. From each titration experiments the two free parameters in the model, the partition coeffecient, K, and the molar enthalpy of partitioning, ΔH, for the lysolipids and fatty acids were calculated as summarized in Tables 2 and 3. A significant higher value for the molar enthalpy of partitioning, ΔH, was found for the fatty acids as compared to the lysolipids although the magnitude of the partition coefficients are comparable. This effect indicates a higher entropy contribution to the driving force for the lysolipid partitioning as compared to the partitioning of the fatty acids into the $DC_{16}PC$ lipid membrane.[43] Interestingly, the molar partitioning enthalpy, ΔH, for $F_{10}A$ is found to be endothermic indicating that the driving force for the lipid membrane partitioning of this short chain fatty acid is predominantly entropic in origin.[43] The hydrophobic force and the gain in

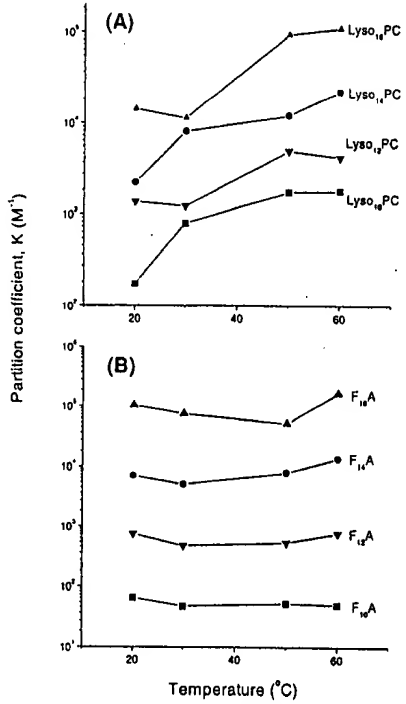

Figure 4. Semilogarithmic plot of the partition coefficient, K, for lysolipids and fatty acids into $DC_{16}PC$ vesicle as a function of temperature. (A) The variation of the partition coefficient, K, for $lyso_n$-PC. (B) The variation of the partition coefficient, K, for F_nA.

entropy due to the release of ordered water molecules surrounding the monomeric hydrophobic chain in the aqueous phase upon transfer of the detergents from the aqueous phase to the membrane phase is expected to contribute in a significant way to the partitioning free energy. However, it is furthermore expected that interactions between the long hydrophobic acyl chains of the detergents and the $DC_{16}PC$ phospholipids constituting the lipid membrane matrix are of importance and most likely give rise to an additional contribution to the enthalpy–entropy balance of the free energy of partitioning. In particular, the degree of mismatch between the acyl chain lengths of the phospholipids and detergents and the interactions between the hydrophobic acyl chains might be considered of importance. In fact, the observed crossover in the enthalpy of partitioning from being exothermic for the transfer of $F_{10}A$ into gel- and fluid-phase lipid membranes (see Table 2) cannot solely be understood on the basis of dehydration and removal of ordered water molecules surrounding the hydrophobic acyl chains of the monomeric detergents in the aqueous phase. This crossover effect strongly suggests that some specific interactions exist between the detergents, e.g., the fatty acids and the phopholipids consituting the lipid membrane matrix. A similar crossover in the chain-length-dependent enthalpy of partitioning of alcohols into phospholipid membranes has recently been reported.[46]

Furthermore, an apparent maximum and high exothermic enthalpy of partitioning is observed for $lyso_{14}PC$ and $F_{14}A$ (see Tables 2 and 3). Whether this effect reflects a high degree of adaption between the acyl chains of the detergents and the phospholipids could be investigated in future studies by a systematic variation of the chain lengths, n, of the phospholipids, DC_nPC, constituting the lipid membrane matrix.

The calculated partition coefficients, K, for both the lysolipids and the fatty acids display a strong dependence on the acyl chains of the detergents. This is manifested as a partition coeffecients that becomes almost an order of magnitude higher when the long acyl chain of the detergents increases by two carbon atoms as clearly seen in the semilogarithmic plots of K as a function of the hydrophobic acyl chain length of the detergents in Figure 3. However, the partitioning of the various fatty acids is found to be independent of the lipid membrane behavior, e.g. the low-temperature gel- and the high-temperature fluid-membrane phases. This is opposite for the lysolipids where the partition coefficients become significantly lower when the lipid membrane vesicles are in the ordered gel state reflecting an intimate relationship between the molecular stucture of the detergents and the phase behavior of lipid membrane. The overall geometrical shape of the lysolipid and fatty acid dertergents varies from nearly cylindrical (F_nA) to cone-shaped ($lyso_nPC$). The large PC-headgroup of the lysolipids therefore imposes additional constraints on the lysolipid partitioning which presumably disfavors a high partitioning of lysolipids into lipid membranes composed of highly ordered lipid acyl chains, e.g., gel-phase membranes at 20 °C and 30 °C. In contrast, the small carboxylic headgroup and the nearly rodlike shape of the fatty acids facilitate the incoporation into an ordered lipid membrane structure. It is therefore expected that the lysolipid partition coefficients, K, into ordered gel-phase lipid membranes are significantly lower as compared to the fatty acid partition coefficients for similar lengths of the hydrophobic acyl chains as clearly reflected by the temperature-dependent partition coefficents for the lysolipids and fatty acids in Tables 2 and 3.

In our calculation we have assumed an equilibrium partitioning of the detergents into both monolayer leaflets of the DC_{16}-PC lipid membrane although the incorporation of the detergents only takes place from the outer aqueous phase of the unilamellar vesicles, when the detergents and vesicles are mixed in the calorimetric sample cell. The equilibrium partitioning assumption for the fatty acids is based on earlier studies showing a very fast fatty acid flip-flop rate from the outer monolayer leaflet to the inner leaflet on the order of milliseconds.[44] However, for the lysoPC a very slow flip-flop rate on the order of several hours has been reported[45] indicating that during the time-scale of the titration experiments, the lysolipids would incorporate predominantly into the outer monolayer leaflet of the membrane. This would lead to an asymmetric lysoPC distribution between the outer and inner leaflets of the 100 nm $DC_{16}PC$ vesicles. Using micropipet techniques, the incorporation of lysolipids into the outer leaflet of large unilamellar vesicles has been reported to be a fast process (rate constant $\sim 0.2\ s^{-1}$) whereas the transfer across the membrane was a significantly slower process (rate constant $\sim 0.0019\ s^{-1}$).[47] In the calculations, we have used the total $DC_{16}PC$ phospholipid concentration, C_L for the different $lyso_nPC$ (n = 10,12,14, and 16) to establish the partition coefficients, K. This might lead to an overestimation by a factor of 2. However, the equilibrium concentration in the outer monolayer leaflet of the lysolipids is expected to be higher than the concentration in the inner leaflet due to curvature constraints imposed by the 100 nm vesicles and the rather large headgroup

of the detergents.[45] The asymmetric lysoPC distribution is furthermore expected to be influenced by the acyl chain length and the overall cone-shaped geometry of the $lyso_nPC$ detergents. However, the quality of the fits (cf. Figures 1 and 2) and the calculated values for the enthalpies of partitioning, ΔH, for the lysolipids are unaffected if the actual $DC_{16}PC$ phospholipid concentration, C_L, is reduced by a factor of 0.5.

Several studies have shown that the activity of lipid membrane associated and interfacially activated enzymes such as PLA_2 is strongly influenced by the lysolipid and fatty acid detergents.[23-25,28,29] It has been shown, that when a certain threshold concentration of the lysolipids and fatty acids are either premixed or accumulated in the lipid membrane, the hydrolysis rate of PLA_2 increases by several orders of magnitude. However, the detailed functional role(s) of the lysolipid and fatty acid hydrolysis products are not fully understood. Several mechanisms have been proposed involving the formation of (non-)equilibrium phase separated lipid domains and an increased affinity and binding of PLA_2 to the lipid membrane.[16,24,28,29] The partition coefficients obtained in the present study suggest new interesting studies to be carried out, where the local lipid membrane concentrations of the various hydrolysis products are controlled and varied in a systematic manner. It certainly would be of interest to determine the threshold concentrations in terms of both exogenously added detergents and the corresponding local lipid membrane concentrations of short- and long-chain detergents required to induce spontaneous activation. Such results would open up for a deeper understanding of the concentration-dependent influence of the detergents on the binding kinetics of PLA_2 to the membrane[28] and the degree of lipid domain formation, which would be strongly determined by the concentration and the mixing properties between the hydrolysis products and the phospholipids constituting the lipid membrane matrix.[10,27]

A deeper understanding of the molecular mechanisms underlying the lipid membrane perturbing and permeability-enhancing effect of lysolipids and fatty acids is of importance in relation to liposomal drug delivery systems[8] as well as for the pharmacological action of anticancer lysolipids.[21] Only a minor permeability-enhancing effect of lysolipids toward fluid-phase lipid membranes has been reported as compared to a significant permeability enhancement toward gel-phase membranes. However, on the basis of values for the partition coefficients established in the present study, it becomes evident that this effect does not correlate with higher concentrations of detergents incorporated into the lipid membrane, when the same global concentrations of the detergents are exogenously added to the aqueous phase.[31] Furthermore, in our measurements of the partition coefficients, we observe a much higher lysolipid partitioning into fluid-phase membranes suggesting different molecular mechanisms involved in the lipid membrane perturbing and permeability-enhancing effect toward gel- and fluid-phase membranes.[31,32] This important result strongly suggests that significantly lower local membrane perturbing concentrations of, for example, lysolipid detergents are required, when the lipid membrane is in the gel phase characterized by highly ordered and tightly packed lipid acyl chains as compared to fluid-phase membranes with highly disordered lipid acyl chain. Therefore, it can be concluded that fluid lipid membranes can incorporate a high amount of detergents without giving rise to any significant perturbation of the lipid membrane barrier properties. In general, the molecular mechanisms involved in the lipid membrane perturbing and permeability-enhancing effects of the detergents are poorly understood although it has been suggested that an increased leakage due to pore formation[20] or an accumulation and desorption of lysolipids from two-dimensional grain boundary regions[8] might be involved. The nonrandom lateral distribution of the detergents in the membrane plane can possibly be investigated using two-dimensional energy transfer techniques that earlier have been used to study small-scale lipid domain formation.[48,49] A phase-dependent membrane perturbing effect has furthermore been reported in more biologically related systems, where it has been observed that the lysis capability of lysolipids toward red blod cell membranes displayed a strong temperature dependence. A significant lysis was observed when the red blood cells were cooled to low temperatures where the lipid membrane part of the cell membrane turned into a gel-like structure suggesting different molecular mechanisms involved in the lysis event of fluid- and gel-state cell membranes.[32]

The partition coefficents for the various lysolipid and fatty acid detergents established in the present study are of interest in relation to a deeper understanding of the effect of global aqueous and local lipid membrane detergent concentrations on functional lipid membrane properties, e.g., the lipid membrane permeability and the activity of lipid membrane-associated enzymes such as PLA_2. In addition, in future titration studies it would be of interest to determine the partitioning and the membrane concentration of fatty acids and lysolipids in more complex lipid membrane systems containing sterols, unsaturated lipids, as well as lipid vesicles premixed with a small molar fraction of either the lysolipids or fatty acids.

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BBA Report

Osmotic dependence of the lysophosphatidylcholine lytic action on liposomes in the gel state

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Key words: Liposome; Lysophosphatidylcholine; Osmolarity; Gel state; Critical lytic concentration; Critical micellar concentration

Multilamellar liposomes of dimyristoylphosphatidylcholine are susceptible to lytic action of lysophosphatidylcholine at the gel state, an effect which is not observed when liposomes are in the liquid crystalline state. The lytic action has been found to be enhanced when liposomes are dispersed in hypertonic solutions. On the contrary, hypotonic solutions decreased the effectiveness of the lysolipid. Shrunken liposomes present surface changes as detected by merocyanine 540 and 1-anilinonaphthalene-8-sulfonic acid which can be ascribed to the spontaneous curvature promoted by shrinkage.

It is known that lysophosphatidylcholines can bind to natural and model lipid membranes inducing morphological changes, cell aggregation, membrane fusion and lysis [1–10]. However, it is unknown whether some of these effects, particularly the lytic actions, are due to monomer or to micelle lysolipid interactions with the membrane and how this effect depends on the gel or liquid crystalline state of the membrane structure. Regardless the degree of fluidity, hydration and packing of bilayers is different in the gel and in the liquid crystalline state, very few attempts have been performed to relate those features to the lysolipid lytic action [6,8]. Hence, in order to get some insight into these problems, we have studied the lytic effect of monomyristoyllysophosphatidylcholine (lyso-PC) on multilamellar liposomes in the gel and in the liquid crystalline state. In addition, the effectiveness of the lysolipid was tested on liposomes suspended either in hypertonic or hypotonic solutions. On such conditions, the surface changes occurred on the liposome surface, monitored by optical and fluorescent probes, significantly affected the lytic effect of lysoPC.

Materials and methods. Dimyristoylphosphatidylcholine (DMPC) and lysoderivates were obtained from Avanti Polar Lipids Inc. 1-Anilinenaphthalene-8-sulfonic acid (ANS), octadecylrhodamine (o-Rh) and merocyanine 540 (MC540) were purchased from Molecular Probes. The purity of the lipids was checked by thin-layer chromatography.

All other chemical were of analytical grade and used without further purification. Water was twice distilled in a standard Milli Q equipment.

Abbreviations: DMPC, dimyristoylphosphatidylcholine; lyso-PC, monomyristoyllyso-phosphatidylcholine; o-Rh, octadecylrhodamine; ANS, 1-anilinonaphthalene-8-sulfonic acid; clc, critical lytic concentration; cmc, critical micellar concentration.

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be stressed that this decrease of absorbance corresponds to the increase of octadecylrhodamine fluorescence. These dilution assays of a self-quenched membrane probe, indicate that absorbance changes are linked to a mixture of lipids. Operationally, the critical concentration at which these processes occur can be defined as the intersection of the two lines depicted in Fig. 1. Comparable clc values were obtained by absorbance and when lysoPC was added either to a sample containing two populations of liposomes, one with and another without octadecylrhodamine or to a dispersion with one population of liposomes labelled with the fluorophore (Fig. 1B).

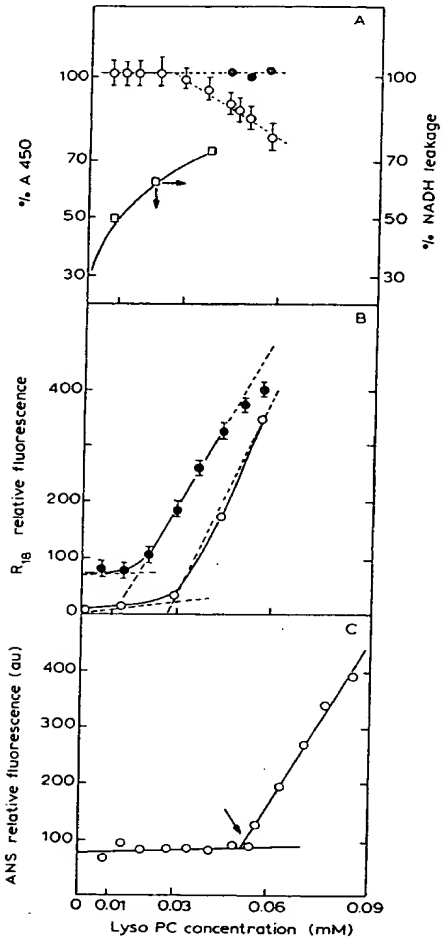

Fig. 1. Effect of monomyristoyllysophosphatidylcholine (lysoPC) on dimiristoylphosphatidylcholine liposomes. (A) Percentage of absorbance change of liposome dispersion above (●·····●) and below (○·····○) their gel-liquid crystalline transition temperature as a function of the lysoPC concentration in the medium. (□) indicates the percentage of NADH leakage from liposomes in the gel state after each lysoPC addition. (B) Changes induced by lysoPC in the relative fluorescence intensity of an homogeneous mixture of liposomes labelled with o-Rh (●) and on another dispersion constituted by two populations of liposomes one with an another without o-Rh in their membrane (○). (C) Critical micellar concentration (cmc) of lysoPC as measured by the fluorescence of an ANS solution. The arrow corresponds to a cmc value of $54 \cdot 10^{-6}$ M (See Table I and Materials and Methods).

TABLE I

EFFECT OF DIFFERENT OSMOLARITIES ON THE CRITICAL MICELLAR CONCENTRATION OF 14:0-LYSOPHOSPHATIDYLCHOLINE

	Buffer Tris (10 mM)	Sucrose (700 mM)	KCl (500 mM)
cmc (mM)	0.050	0.039	0.028

The critical micellar concentration of lysoPC, estimated as explained in Materials and Methods was obtained at the different osmolarities in sucrose, KCl and buffer Tris solutions (Fig. 1C and Table I). Different osmolarities affect the critical micellar concentration of the 14 : 0 lysophosphatidylcholine in the following order: buffer 10 mM Tris-HCl (pH 7), 0.050 mM; 0.7 M sucrose, 0.039 mM and 0.5 M KCl, 0.028 mM. Since in the experiments represented in Figs. 1A and 1B, the clc of lysoPC was below such concentrations ($\leqslant 0.020$ mM), this compound would exert its lytic effect when in the monomeric form.

Another consequence of the lysoPC addition was the release of trapped NADH. This release took place only when liposomes were in the gel state. However, in this case, a 50% release was obtained at lower lysoPC concentration compared to those found using either fluorescence or absorbance methods. Such discrepancy could be due to an incorporation of the lysocompound to the liposomes, followed by a change in the barrier properties, without the disruption of the liposomes. Another possibility could be a NADH leakage, occurring in a liposome population composed of smaller particles which can not be detected by the optical methods. As it is well-known the coarse lipid dispersion has a wide size distribu-

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Review

Mixed micelles and other structures in the solubilization of bilayer lipi membranes by surfactants

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Abstract

The solubilization of lipid bilayers by surfactants is accompanied by morphological changes of the bilayer and th emergence of mixed micelles. From a phase equilibrium perspective, the lipid/surfactant/water system is in a two-phase are during the solubilization: a phase containing mixed micelles is in equilibrium with bilayer structures of the lamellar phase. Ir some cases three phases are present, the single micelle phase replaced by a concentrated and a dilute solution phase. In th case of non-ionic surfactants, the lipid bilayers reach saturation when mixed micelles, often flexible rod-like or thread-like start to form in the aqueous solution, at a constant chemical potential of the surfactant. The composition of the bilayers alsc remains fixed during the dissolution. The phase behavior encountered with many charged surfactants is different. The lamellar phase becomes destabilized at a certain content of surfactant in the membrane, and then disintegrates, forming mixed micelles, or a hexagonal phase, or an intermediate phase. Defective bilayer intermediates, such as perforated vesicles, have been found in several systems, mainly with charged surfactants. The perforated membranes, in some systems, go over into thread-like micelles via lace-like structures, often without a clear two-phase region. Intermediates in the form of disks, either micelles or bilayer fragments, have been observed in several cases. Most noteworthy are the planar and circular disks found in systems containing a large fraction of cholesterol in the bilayer. Bile salts are a special class of surfactants that seem to break down the bilayer at low additions. Originally, disk-like mixed micelles were conjectured, with polar membrane lipids building the disk, and the bile salts covering the hydrophobic rim. Later work has shown that flexible cylinders are the dominant intermediates also in these systems, even if the disk-like structures have been re-established as transients in the transformation from mixed micelles to vesicles.

Keywords: Solubilization; Lipid bilayer; Mixed micelle; Phase diagram; Liposome; Perforated bilayer; Intermediate phase; Mesh phase; Disk

Abbreviations: C_xE_y, $CH_3(CH_2)_{x-1}(OCH_2)_yOH$; $C_xTAB(C)$, alkyltrimethylammonium bromide(chloride) x C in alkyl chain; cmc, critical micellar concentration; cTEM, cryotransmission electron microscopy; DG, dodecylglucoside; DMPC, dimyristoylphosphatidylcholine; DM, dodecylmaltoside; DOPE, dioctadecylphosphatidylethanolamine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; EPC, egg phosphatidylcholine; GMO, glyceryl monooleate; HG, heptylglucoside; H_I, hexagonal phase; L_1, L_2, L_3, isotropic liquid phases (micelle phase, reversed micelle phase, sponge phase); L_α, lamellar liquid crystalline phase; OG, octylglucoside; PEG, polyethylene glycol; Q_I, isotropic cubic phase; R_{sol}, R_{sat}, surfactant to lipid molar ratio at complete solubilization of the bilayer, and at saturation of the bilayer by surfactant, respectively; SDS, sodium dodecylsulfate; SANS, small angle neutron scattering; SAXS, small angle X-ray scattering; Triton X-100, [*p*-(1,1,3,3-tetramethylbutyl)phenyl]poly(oxyethylene)

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1. Introduction

The dissolution of a bilayer lipid membrane by addition of surfactants finally results in mixed micelles containing both polar lipids and surfactants. By removing surfactant from the mixed structures, for instance by adsorption, dialysis or, if the surfactant is sufficiently soluble, just by dilution, the lipid bilayer may be reconstituted and closed into a liposome. The equilibrium and transient structures encountered in lipid–surfactant mixtures, and the kinetics of the dissolution and of the closure to vesicles are important issues, and have attracted considerable interest [1,2]. In this review we will focus on the structures and discuss them in relation to the equilibrium properties and phase relations. These aspects of the equilibrium state of the systems are contained in the phase diagrams. Unfortunately, only few systems comprising polar lipids and surfactants in water have been studied in sufficient detail. We shall review the information available, and let that knowledge guide the discussion of the structures in the systems.

The dissolution of bilayer membranes is usually studied in an excess of solvent, either water or an electrolyte solution, starting from an aqueous dispersion of the lamellar phase in the form of liposomes (or vesicles), and ending with mixed micelles belonging to the L_1 phase. A variety of structural transitions may occur in the process. Often non-equilibrium structures, such as liposomes, are kinetically trapped, and have a size and morphology depending on the history of the sample. Sometimes slow changes can be followed over hours or weeks, but in other cases no changes are observed even after years of storage, at least nothing that can be distinguished from transformations due to a slow chemical breakdown.

2. Systems of polar lipid, surfactant, and water

A phase diagram summarizes extensive information about the equilibrium state of a system. The (quasi) three-component systems considered here are usually presented in the form of Gibbs triangles, at a constant temperature. A complete diagram should contain tie lines in the two-phase areas, showing the equilibrium compositions of the two phases involved, but few systems have as yet been studied in such detail.

2.1. Phospholipids and non-ionic surfactants

The solubilization of bilayer membranes by various non-ionic surfactants has attracted much attention, in particular using Triton X-100 ([*p*-(1,1,3,3-tetramethylbutyl)phenyl]poly(oxyethylene) with an average of 9.5 oxyethylene groups), octylglucoside (OG), and $C_{12}E_8$ and related polyethylene glycol (PEG) surfactants. Such surfactants are commonly employed in membrane research, e.g. in the extraction and reconstitution of membrane proteins. In spite of that, fundamental phase studies of such systems are sparse. In fact, except for a partial diagram of the system egg phosphatidylcholine (EPC)/OG/water presented in a PhD thesis [3], soybean PC/Triton X-100/water seems to be the only system of a non-ionic surfactant and phospholipid for which a detailed study of the phase behavior has been published [4]. Although neither the polar lipid nor the surfactant are single pure substances, we have to rely on this system for general considerations.

2.1.1. Phospholipid/Triton X-100/water

The surfactant forms a normal sequence of phases in water: the micelle phase L_1 is followed by a hexagonal phase, and at high concentrations of the surfactant, which is fluid at room temperature, there is a L_2 phase. Each of these phases can solubilize lecithin to some extent, at most about 10% in the L_1 phase (Fig. 1). The lamellar phase, L_α, of the phospholipid readily takes up Triton X-100, and then swells in water to a maximum of 50% water. At lower temperatures (278 K) the lamellar phase extends to the surfactant–water axis, i.e. the surfactant itself forms a lamellar phase in water, which is connected to the lamellar phase of the lecithin.

Solubilization of bilayers is usually studied in dilute liposomal dispersions. We start then at a point, say A, on the lecithin–water axis, where the lamellar phase is dispersed in excess water. On addition of surfactant the composition follows the line AB in the two-phase area, and enters the mixed micelle region at point B. In the two-phase area the concentration of surfactant increases in both phases until

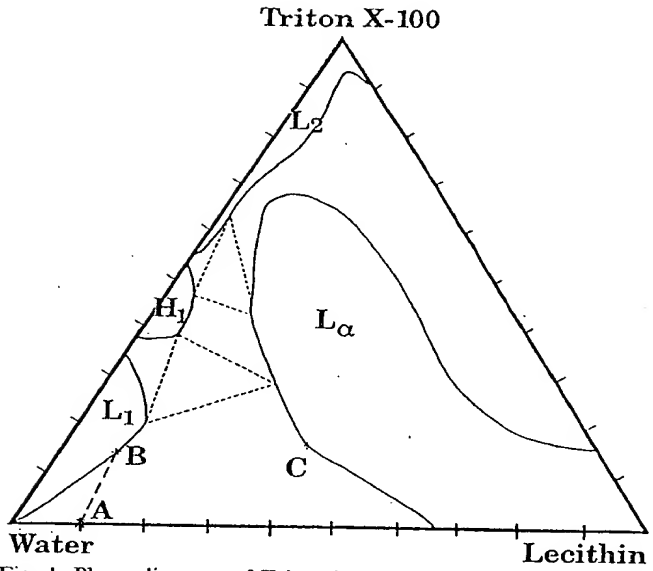

Fig. 1. Phase diagram of Triton X-100/soybean lecithin/water at 23°C. Four one-phase areas are shown: L_1 (micelle solution), H_1 (hexagonal phase), L_2 (reversed micelles) and L_α (lamellar phase), and two three-phase triangles. The line AB indicates total compositions followed on addition of surfactant to a sample of lecithin in water at point A. Point C indicates a possible composition of the bilayer in equilibrium with mixed micelles. Adapted from [4] with permission. Copyright 1989 Academic Press.

the critical micellar concentration (cmc) for formation of mixed micelles is reached. This concentration is necessarily lower than the cmc of the pure surfactant. In the system of Fig. 1 the cmc is close to the water corner and not marked. At higher concentrations of surfactant, saturated mixed micelles are in equilibrium with the mixed lamellar phase. The saturation limit of the L_1 phase is almost a straight line extending to the water corner (or more precisely to the cmc), implying that the composition of the micelles in equilibrium with the lamellar phase is almost constant (37% lecithin by weight is obtained from the 'slope' of the line, or about 32 mol%, corresponding to a molar ratio of surfactant to lipid of 2.1). The concentration of surfactant in the intermicellar aqueous solution is then also almost constant and so is the composition of the bilayer. Although the tie lines in the two-phase area have not been determined, it is a fair guess that when micelles have formed the tie lines all end at a bilayer composition close to point C on the L_α border, and start on the straight line part of the L_1 saturation curve. The change of the bilayer composition up to point C occurs as the surfac concentration in the aqueous phase, on the first ditions, increases from nothing to the mixed cm

The solubilization process can then be describe follows. The surfactant is distributed between s tion and membrane up to a point (close to C) w the membrane is 'saturated' with surfactant, surfactant/lipid molar ratio, R_{sat}. Further addi of surfactant results in the formation of mixed celles of an almost constant composition, given b ratio R_{sol}. At point B, the last piece of membran dissolved when the system enters the L_1 phase. T description of the process is close to that of 'three-stage model' usually assumed, where the c stant compositions of the mixed micelles and 'saturated' bilayers are taken for granted [2,5- From the three-component phase diagram, we c clude that constant compositions of bilayer mixed micelles during the dissolution require t the tie lines in the L_α/L_1 two-phase area connec single point on the limit of the lamellar phase, w points on the linear part of the L_1 border that tends to a point close to the water corner. Furth more, the diagram shows that in the case of Trit X-100, the composition of the bilayer during the s ubilization is not determined by a saturation of t bilayer – the bilayer can accept much more of t surfactant – but by the onset of micelle formatior

Many detailed studies of the dissolution have be made by measurement of turbidity or scattered lig intensity. The changes often follow a general patte and it is possible to identify a few characteristic fe tures of the solubilization curve. By measuring t surfactant concentration required to reach these fe tures at different lipid concentrations, both the co centration of free surfactant and the composition the aggregate can be determined. These paramete are assumed constant. Referring to the phase di gram in Fig. 1, it is evident that characteristic poin that can be identified in this way correspond to cor positions on the tie line connecting the 'mixed cm and the point C, and the compositions defining tl saturation boundary of the L_1 phase.

Paternostre et al. [8] studied the solubilization EPC liposomes by Triton X-100, OG, and sodiu cholate, using both turbidity monitoring and NM methods. They found for Triton X-100 a molar rati of surfactant to lipid of $R_{sol} = 0.64$ in the saturate

bilayer, and $R_{sat} = 2.5$ in the saturated mixed micelles. Dennis [5], using direct chemical analysis of the phases separated by centrifugation, obtained very similar results, 0.63 and 2.4, respectively, at higher concentrations of lipid (2.2%) and surfactant. It can be noted that Paternostre et al. and Dennis prepared the samples in very different ways. The former made liposomes by reversed phase separation, whereas the latter normally used a gentle homogenizer to mix the samples. Point C in Fig. 1 corresponds to a surfactant/lipid ratio of 0.54, and the slope of the L_1 saturation line to 2.2. These latter values refer to soybean lecithin, which may explain the deviation from the solubilization studies using EPC.

The morphologies of the bilayers and the micelles during the solubilization process were studied in cryotransmission electron microscopy (cTEM) investigations of the same system [9]. The bilayers were originally present as small sonicated vesicles, which grew after a certain amount of surfactant had been added. The large vesicles opened and turned partly into irregular and curved bilayer flakes at higher concentrations of surfactant, and at the same composition long cylindrical micelles started to appear. Similar results have been reported with other detergents [10,11]. A typical series of micelle and bilayer morphologies is shown in Fig. 2. If the three-stage model were perfectly valid, one would expect that when mixed micelles and saturated bilayers are simultaneously present, they would have morphologies that were independent of the total composition. The cTEM investigations, however, and also the detailed analysis of binding isotherms [1,12], suggest a more gradual transition. Several reasons for such a behavior may be envisioned. (i) Small changes in the composition of the mixed micelles and of the bilayer may occur that can give large morphological effects. (ii) Interactions between the bilayer structures, or between micelles, or between micelles and bilayers, may give appreciable differences in the morphologies of both bilayer structures and micelles. (iii) During

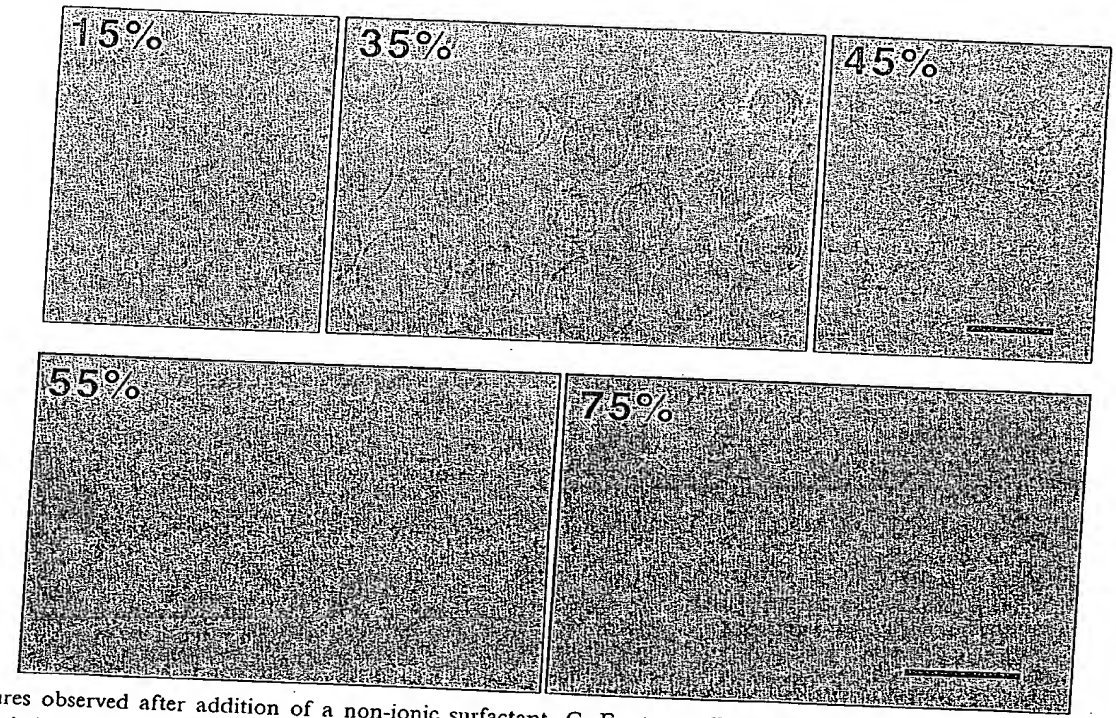

Fig. 2. Structures observed after addition of a non-ionic surfactant, $C_{12}E_8$, to small, sonicated EPC vesicles. The mol% surfactant in the dry sample is indicated; the EPC concentration was 1.2 mM. At 35% the vesicles have grown to their maximum radius. With larger addition of surfactant long thread-like micelles are first formed, followed at higher surfactant concentrations by shorter threads and finally globular micelles. Reproduced and adapted with permission from [11]. Copyright 1991 Academic Press.

solubilization the system may become trapped in different non-equilibrium states.

Points (ii) and (iii) need some comments. (ii) When solubilization curves are recorded at different lipid concentrations an increased turbidity is often observed at the higher concentrations (which in itself is a source of error), due to attractive interactions between the liposomes that give rise to clustering, and sometimes to a secondary transformation into large and multilamellar structures. Ultimately, macroscopic phase separation may occur. The attraction may partly be an effect of depletion forces from the mixed micelles. (iii) With respect to the equilibration time, there are several stages. The redistribution of surfactants between the aqueous solution and the bilayers can be expected to be fast (at least to the outer half of the bilayer, the flip-flop or other penetration of the membrane may be very slow, in particular for charged surfactants [2]). The equilibration of the mixed micelles and the solubilization of a bilayer in excess surfactant have also been found to proceed relatively fast in studies of micelle relaxation processes [13], as well as in direct measurements of the solubilization times of bilayers [2,9,11]. The morphology of the bilayer structures, however, can be expected to evolve very slowly towards the most stable ones – probably often represented by a small chunk of lamellar phase. As a secondary effect from the slow change of the bilayer arrangements a redistribution of surfactant may occur, possibly in turn having a small effect also on the composition of the mixed micelles.

Normally, however, we expect that the micelles observed represent equilibrium at the solubilization limit of the L_1 phase, and that the composition of the bilayers is close to the equilibrium composition, even if the bilayers may take on a variety of non-equilibrated structures depending on how the samples were prepared.

2.1.2. Alkylglucosides and other non-ionic surfactants

OG has been utilized extensively in biochemical membrane research to solubilize bilayer membranes and for reconstitution experiments, and the interactions of OG with bilayers have been studied thoroughly [1–3,6–8,14–17]. A range of other sugar surfactants have also been examined, with a variation of both alkyl tail length and headgroup [18–23], for example dodecylmaltoside (DM). Phase separatic and secondary aggregation phenomena during t solubilization are often noticed for this class of su factants [14]. Such complicating phenomena were e amined in detail recently, in a series of papers fro Ollivon and his group [15,21,22]. The main objecti of these studies was to investigate an enzymatic pr cedure for the formation of vesicles from mixed m celles of polar lipids and DM. By enzymatic hydr lysis of the maltoside group, first to glucoside, D and eventually to dodecanol and glucose, the lar polar headgroup of the maltoside is converted into much smaller headgroup, so that the surfacta packing parameter, $v/a_{head}l_0$, approaches unity, in the case of the alcohol exceeds unity. A transiti from the mixed micelles with positive spontaneo curvature to the balanced state of the bilayer is t result.

To clarify the details of the process, dipalmito phosphatidylcholine (DPPC)–DG mixtures were i vestigated in excess buffer, at temperatures above t gel-to-liquid crystalline transition of the mixtures, several methods, and found to form closed bilay vesicles at DG/DPPC molar ratios up to 1.8; abo this concentration discoid structures also appear (but not thread-like micelles) [21]. Solubilization the mixed bilayers with DM gave complex turbid curves with a number of characteristic points. Me surement at different total concentrations resulted the pseudo-ternary phase diagram, in excess buff that is reproduced in Fig. 3 [21]. The morphologies the different areas were determined by X-ray stud and freeze-fracture electron microscopy. In the a sence of DG, DM solubilizes DPPC bilayers shown on the y-axis in the figure. The lamellar pha can take up DM to a molar ratio of 0.5 before m celles appear (step III), and the last bilayers are sol bilized at a molar ratio of 1.5 (step VII). With D added, DM/DPPC increases slightly for step III, a strongly for step VII, indicating that DG is incorp rated into the bilayer without much destabilizatio

The complexity of the turbidity curve and t phase diagram is due to secondary aggregation a phase separation. Two types of processes seem to involved: liquid–liquid phase separation of the mix micellar solutions, similar to the well known clou ing of non-ionic surfactants, and attractive inter tions between the vesicles in dispersion when t

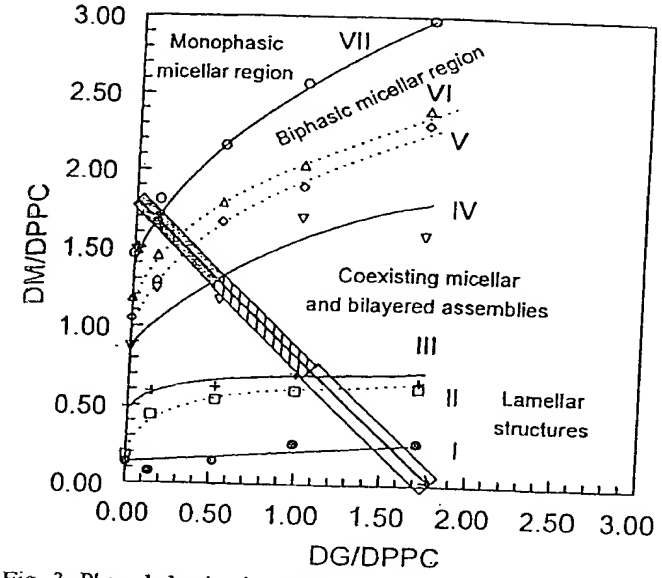

Fig. 3. Phase behavior in excess buffer (145 mM NaCl, 10 mM HEPES) of DPPC/DG/DM at 37°C. The solid lines delimit main phase transitions (observed by SAXS) and the dotted lines secondary aggregation transitions. The crossing arrow indicates the path with a constant sugar/lipid ratio of 1.8, from DM/DPPC mixed micelles, over two-phase regions with micelles, and micelle water, to the three-phase area with two micellar and a lamellar phase, and then lamellar phases in equilibrium with dilute L_1; the final composition without DM and 1.8 DG/DPPC is a pure lamellar phase in buffer. Reproduced and adapted with permission from [21]. Copyright 1998 American Chemical Society.

surfactant is added, leading to agglomeration and at high lipid concentrations to macroscopic phase separation [21]. The latter process has been observed also with Triton X-100, as remarked above. From the results and discussion presented in [21] and from private communication with Lesieur and Ollivon, the following picture emerges.

Up to line III in Fig. 3, there are different lamellar phases in equilibrium with a dilute L_1 phase, denoted excess water in the reports. Between lines III and V there is a coexistence region of mixed micelle and bilayer, which in reality is a three-phase area of lamellar phase and two L_1 phases, one mixed micellar, the other very dilute. After line V no lamellar phase is present, and lines V–VII delimit a region with the two micellar phases in equilibrium, and beyond line VII there are only mixed micelles.

In the PhD thesis of Beugin-Deroo [3] a similar phase behavior of heptylglucoside (HG) and OG/EPC/water was demonstrated. In this system, it was shown by centrifugation of samples and subsequent X-ray examination of the separated phases that at EPC concentrations close to saturation of the mixed micelles, a liquid–liquid phase separation occurred into a dilute phase containing no or few micelles, and a more concentrated mixed micellar solution. In the phase diagram, the first part of the solubility limit of the L_1 phase was thus replaced by a narrow two-phase area. At higher lipid-to-surfactant ratios, the samples contained three phases. In addition to the concentrated and dilute micelle phases, there was a new phase of lamellar structure, according to the small angle X-ray scattering (SAXS) study. A turbidity peak in the solubilization curve corresponds to compositions within the three-phase triangle. Solubilization of the bilayer with these surfactants thus brings the system into a three-phase triangle, with one corner fixed on the lamellar phase border, at the appropriate R_{sat}, and the other two being the endpoints of the narrow two-phase area, one close to the mixed cmc, and the other at a higher concentration of lipid. The difference of this case compared to the Triton X-100 described above is that the narrow two-phase strip has closed to the L_1 saturation border in the Triton case; a two-phase system is replaced by a pseudo-two-phase system of mixed micelles in aqueous solution.

PEG surfactants like Triton X-100 have a cloud point, an upper consolute temperature, that is expected to be lowered by mixing in polar lipids like EPC. It would be interesting, therefore, to examine the Triton X-100 phase behavior, with EPC added, at higher temperature to see if clouding would occur, and if it would give rise to a phase behavior similar to that of OG (or HG) and EPC. OG itself, according to the studies of Kameyama and Takagi [24], does not have an upper consolute temperature; on the contrary, the light scattering studies show that the virial coefficient is negative at low temperature and positive at high; the zero point of the virial coefficient is close to 40°C, so that if anything, the attractive interactions favor phase separation at low temperatures. The issue is not yet sufficiently clarified.

In a study using higher lipid concentrations a gelling of the solutions was reported [20] at a ratio of

DM to phospholipid around 1.4–1.5, close to the end of the bilayer-to-micelle transition region ($R_{sol} \approx 1.6$ in this case). (Similar findings were discussed earlier for EPC/OG [14].) cTEM micrographs reveal that very long thread-like micelles coexist with a few vesicles and other bilayer structures in this region. The micrographs also show that substantial changes of the bilayer morphologies occur at lower surfactant concentrations, before the thread-like micelles appear, and it is possible that the turbidity starts to decrease due to such morphological changes, already before the micelles form.

In summary, the fact that the results from DM, as well as some other non-ionic surfactants, are not fully accounted for by the simple three-stage model is probably mainly due to secondary aggregation phenomena and phase separation. There is also evidence, however, from cTEM micrographs and the rheology of the solution [20], that the micelle morphology changes within the transition region, before all bilayers have disappeared. The maximum viscosity and the so-called Weissenberger effect indicate that the thread-like micelles are particularly long and entangled below R_{sol}. It is possible that an interaction between bilayers and micelles is important for the solution properties, or that non-equilibrium effects can play an important role.

The effect of OG on different polar lipids was recently investigated [15]. DPPC, DOPE, and glyceryl monooleate (GMO) in excess water form a L_α phase, a reversed hexagonal, H_2 phase, and a reversed cubic bicontinuous phase, C_D, respectively. It was nicely demonstrated that the addition of OG increases the curvature of the structures somewhat, so that the reversed structures of GMO and DOPE were transformed into L_α bilayers, and that DPPC remained in that state (with a reduction of the gel-to-liquid-crystal transition temperature).

2.2. Polar lipids and ionic surfactants

The phase behavior of polar lipids and ionic surfactants, in water or in salt solution, has been better studied than that of non-ionic surfactants. It is mainly phospholipids and glycerol monoalkanoates, together with bile salts or cationic surfactants, that have attracted attention; much less is known of normal anionic surfactants. We consider first alkyltrimethylammonium surfactants and polar lipids, and d cuss the bile salts in a separate section.

2.2.1. Dimyristoylphosphatidylcholine (DMPC)/ $C_{16}TAB$/water

This was for a long time the only system of tl type that had been thoroughly studied [25]. The mc conspicuous feature of the diagram (Fig. 4) is t enormous swelling of the lamellar phase that acco panies charging of the bilayer. Already at an ad tion of 1% of $C_{16}TAB$ the lamellar phase swells o to more than 80% water. The maximum swellir which is not easy to quantify experimentally but e ceeds 99% water, is obtained at molar ratios of su factant to lipid in the range 0.8–1.2. When the su factant/lipid ratio exceeds $R_{sat} \approx 1.9$ mixed micell form, with a composition corresponding to a surfa tant/lipid ratio of $R_{sol} \approx 2.8$, both the saturation lin of the L_1 phase and the lamellar phase border a linear, defining constant surfactant/lipid ratios. this system, therefore, it seems to be the bilayer cor position that limits the stability.

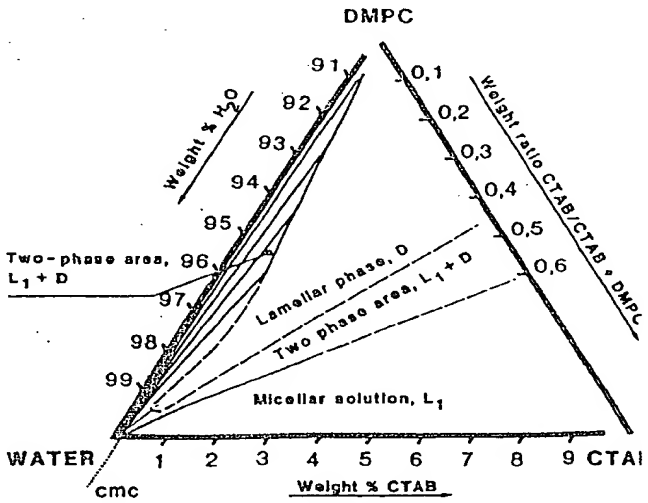

Fig. 4. Phase diagram of $C_{16}TAB$/DMPC/water at 30°C, tl water corner. The lamellar phase of DPPC swells from abo 40% water without $C_{16}TAB$, to a maximum of 99%. On tl surfactant-rich side, $C_{16}TAB$ micelles can solubilize DMPC mixed micelles to a weight ratio 0.67 lipid to surfactant, ind pendent of the total concentration, whereas stable vesicle di persions are formed on surfactant poor side. Note that the su factant is at the right-hand corner in this diagram, instead the top corner. Reproduced with permission from [25]. Cop right 1982 Elsevier Science.

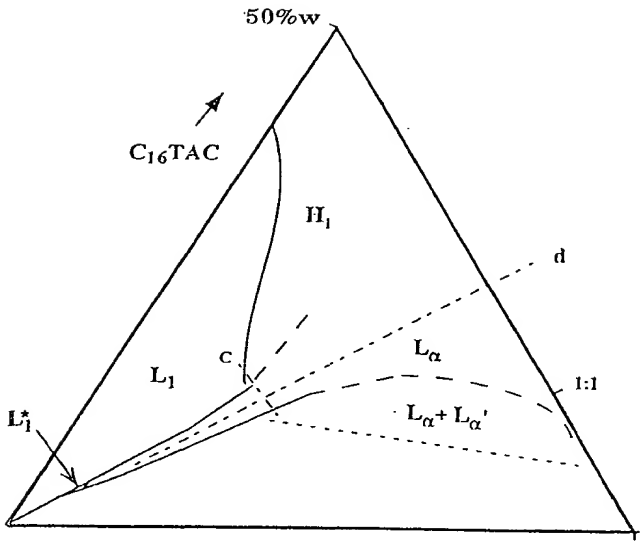

Fig. 5. The system $C_{16}TAC$/EPC/0.100 M NaCl at 25°C. The swelling of the lamellar phase is retained in a narrow tongue, compare Fig. 4. A new flow-birefringent phase L_1* appears. The two-phase area between L_1 and L_α, and between the hexagonal phase H_1 and L_α, is seemingly suppressed. Low addition of surfactant yields two lamellar phases in equilibrium, L_α and L_α'. Reproduced and adapted with permission from [27]. Copyright 1997 American Chemical Society.

On the surfactant-rich side of the lamellar phase, the two phases at equilibrium are the saturated lamellar phase and the mixed micelles. In the surfactant-poor region, a less strongly swollen lamellar phase is in equilibrium with a surfactant solution at a concentration below the cmc (1.3 mM). Vesicle dispersions form easily from the swollen lamellar phase, and the vesicles are stabilized electrostatically towards fusion [26]; charged lipids promote vesicle formation. The maximum swelling limit of the lamellar phase could not be determined precisely. Reliable X-ray results were obtained only at water contents below 80%, which is a level reached already on addition of about 1% of $C_{16}TAB$. It is difficult determine the transition point from a strongly swollen, lamellar phase, with an average distance of maybe 400 nm of water between the undulating bilayers, to a dispersion containing very large bilayer vesicles [25].

When solubilization studies are performed with ionic surfactants, it is not only the charged surfactant that is added, but also its counterion. The electrostatic interactions are most important on the first additions of the surfactant, and are then gradually screened. It is of interest to study the phase behavior with the electrostatic screening kept more nearly constant. Fig. 5 presents a phase diagram of EPC/$C_{16}TAB$/0.100 M NaCl [27]. The swelling of the lamellar phase is reduced on the surfactant-poor side, so that more surfactant has to be added to get an effect. The maximum swelling is reached in a narrow tongue, which extends to about 94% water, centered around a molar ratio of surfactant to lipid of 2.1. With more surfactant the lamellar phase goes over into the micellar phase, without any discernible two-phase area, at a surfactant/lipid ratio of 2.5. A new liquid phase, denoted L_1* in Fig. 5, occupies the tip of the tongue. L_1* is an isotropic (but flow-birefringent) phase, which was not studied in detail. On the other side of the tongue, two lamellar phases are in equilibrium, the one is swollen and surfactant-rich, the other with less surfactant and limited swelling. Similar phase equilibria between two lamellar phases were observed and discussed for other systems containing a mixture of a zwitterionic lipid and a charged surfactant [28,29]. In the EPC/$C_{16}TAC$ case the swollen tongue was shown by extensive SAXS and NMR studies to contain a lamellar phase with water-filled defects [27,30] that on dispersion produces perforated vesicles. In fact, it was just the discovery of such vesicles that prompted the closer inspection of the phase behavior in this system [31].

The turbidity changes observed during the solubilization process, starting with a dilute sample of small unilamellar lecithin vesicles and adding surfactant, is distinctly different with $C_{16}TAC$ or $C_{16}TAB$ than with non-ionic surfactants. Without salt, no changes were observed before the final dissolution into mixed micelles, not even when very small sonicated vesicles were employed at the starting point. In 0.100 M NaCl, on the other hand, the turbidity change indicates a growth similar to that observed with e.g. $C_{12}E_8$, but in this case the change is slow and continues for about 24 h. Micrographs from a cTEM investigation are shown in Fig. 6, where the most unusual feature is the perforated bilayers, which emerge slowly, and are found both closed to vesicles and as open flakes [31].

The cTEM investigations are normally made on very dilute systems, and all micrographs shown

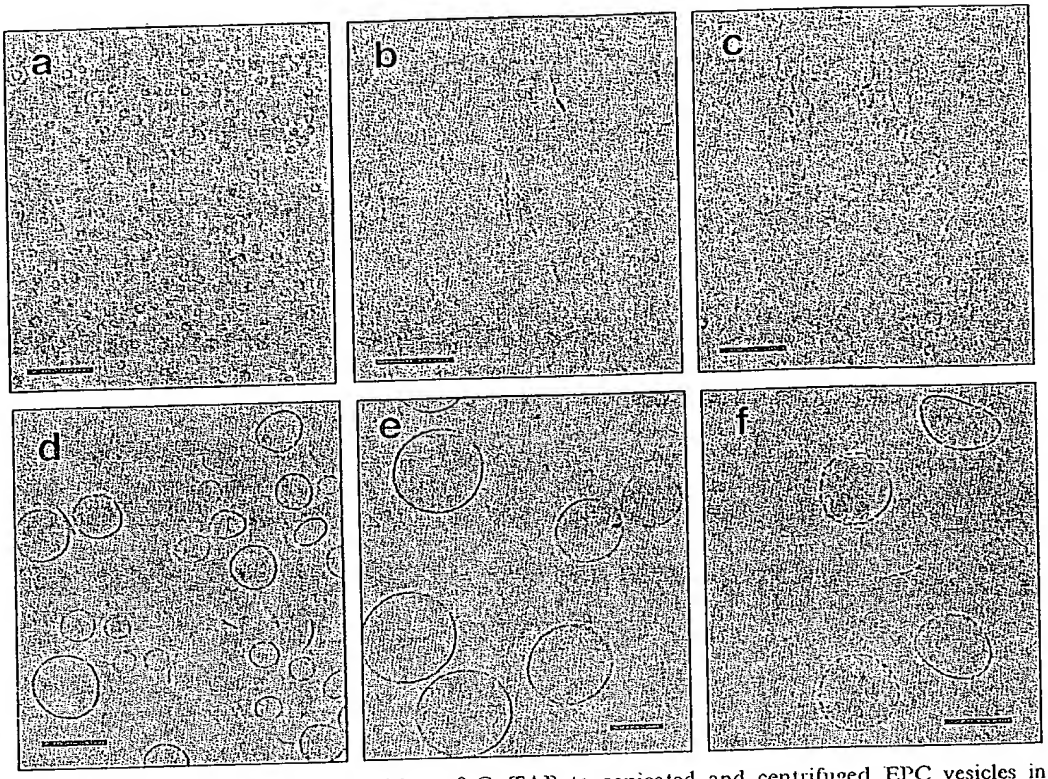

Fig. 6. A sequence of cTEM micrographs from addition of C_{16}TAB to sonicated and centrifuged EPC vesicles in 0.100 M NaC (a) The initial preparation. (b) Lace-like nets and entangled thread-like micelles are found 24 h after addition of 75% C_{16}TA((c) Thread-like micelles, 80% C_{16}TAC, 24 h. The sequence d–f shows structures found 15 min, 3 h, and 24 h, respectively, after add tion of 60% C_{16}TAC. Note in f that even the rim of the vesicles looks perforated. Bar: 100 nm. Reproduced and adapted with pe mission from [31]. Copyright 1993 Academic Press.

here belong to samples that contain only about 1% of lipids and surfactants. The lamellar phase was imaged only in dispersions, therefore. Micrographs of freshly diluted samples prepared from the lamellar phase and the adjoining L_1 phase are also shown in Fig. 6 [30]. There is no clear two-phase area separating L_α and L_1, and the micrographs likewise seem to indicate a gradual transition with increasing surfactant concentration, from perforated bilayers via more loosely woven lace-like structures, to thread-like entangled micelles, and finally shorter rod-like, eventually globular, mixed micelles. No change of the vesicles was observed at low surfactant additions, until the concentration where growth starts. Since growth was slow in this case, it was possible to capture some intermediates during the growth process. It appears that the vesicles first open to discoid structures, which presumably grow by fusion, and finally close again to large vesicles, all this within 1 h. Thereafter the perforated texture slowly emerg and is fully evolved after some 10 h [31].

This process deviates from the three-stage mod where there is a clear transition region from bilaye to mixed micelles. Investigations where the tail leng and headgroup of the ionic surfactant and the ty of polar lipid have been varied, will be discuss next.

2.2.2. *EPC/C_{12}TAC/0.100 M NaCl*

The phase behavior and solubilization characte istics described for the C_{16}TAC system change dr matically when the chain length of the surfactant reduced (Fig. 7) [30]. The tongue towards the aqu ous corner is reduced giving a maximum swelling about 70%. Now two-phase regions are present b tween the lamellar phase and L_1 and the hexagor phase, H_1. The lamellar phase was free from wat filled defects, as evidenced from the SAXS and NM

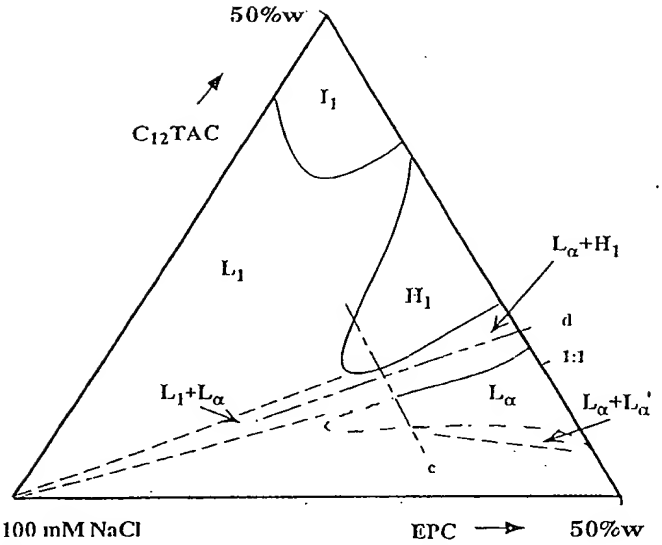

Fig. 7. $C_{12}TAC$/EPC/0.100 M NaCl at 25°C. Compared to the corresponding $C_{16}TAC$ system in Fig. 5, the lamellar phase swells much less, and there are now clear two-phase regions between L_α and L_1 or H_1. Reproduced and adapted with permission from [30]. Copyright 1997 American Chemical Society.

measurements. The extensive swelling in the EPC/$C_{16}TAC$ system, even in the presence of salt, indicates that relatively long-range repulsive forces are present, other than electrostatic. Helfrich undulation forces [32] are the probable source of the repulsion. The reason for the large swelling with $C_{16}TAC$ as surfactant, compared to $C_{12}TAC$, would thus be that the perforated bilayers are much more flexible than the compact bilayers, and give large amplitude undulations.

The solubilization process is illustrated by the micrographs in Fig. 8, and is seen to occur in a typical three-stage sequence: incorporation of surfactant in the bilayers gives vesicles of varying size and shape, mixed micelles start to form, in this system thread-like micelles, that coexist with vesicles until all bilayers are solubilized. As more surfactant is added, the micelles finally go over into globular form (not shown). Most of the vesicles found in the two-phase area, together with thread-like micelles, had disrupted bilayers [30].

2.2.3. *GMO/$C_{16}TAC$/water or brine*

GMO is probably best known for the fact that it exposes a cubic phase to excess water or brine. The cubic phase is of the reversed type, i.e. the mean curvature of the lipid monolayers is slightly negative. When a charged surfactant is added the spontaneous curvature first gets balanced at zero, forming planar bilayers in a lamellar phase that on further addition of surfactant decomposes into mixed micelles, sometimes via defect bilayers. As shown in the phase diagrams of Fig. 9, for the system GMO/$C_{16}TAC$/water (brine), the charging of the bilayers also gives rise to an strong swelling of both the lamellar phase and the

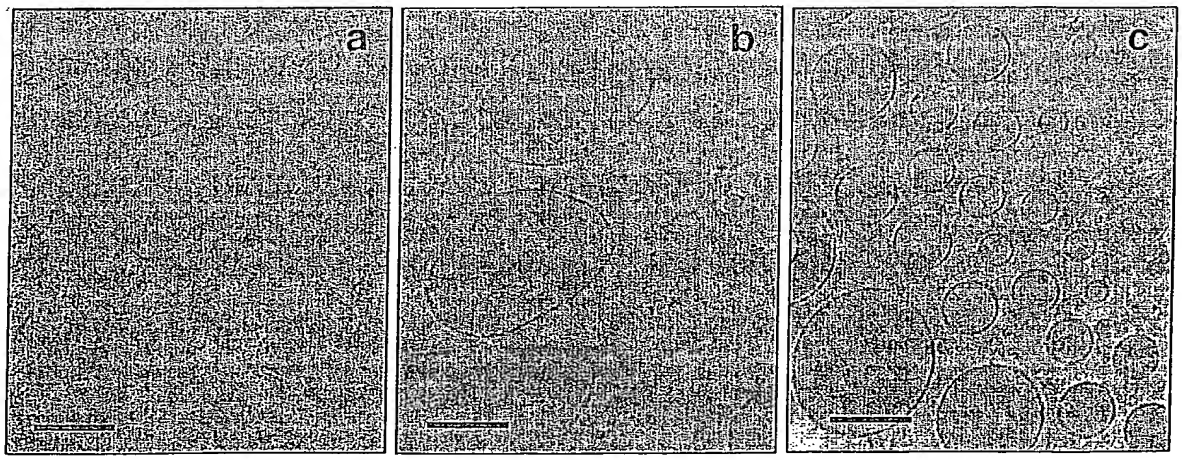

Fig. 8. In the solubilization of EPC by $C_{12}TAC$, large vesicles (c) dissolve into threads, coexisting (b) with vesicles having disrupted bilayers, and finally make thread-like micelles (a). Reproduced and adapted with permission from [30]. Copyright 1997 American Chemical Society.

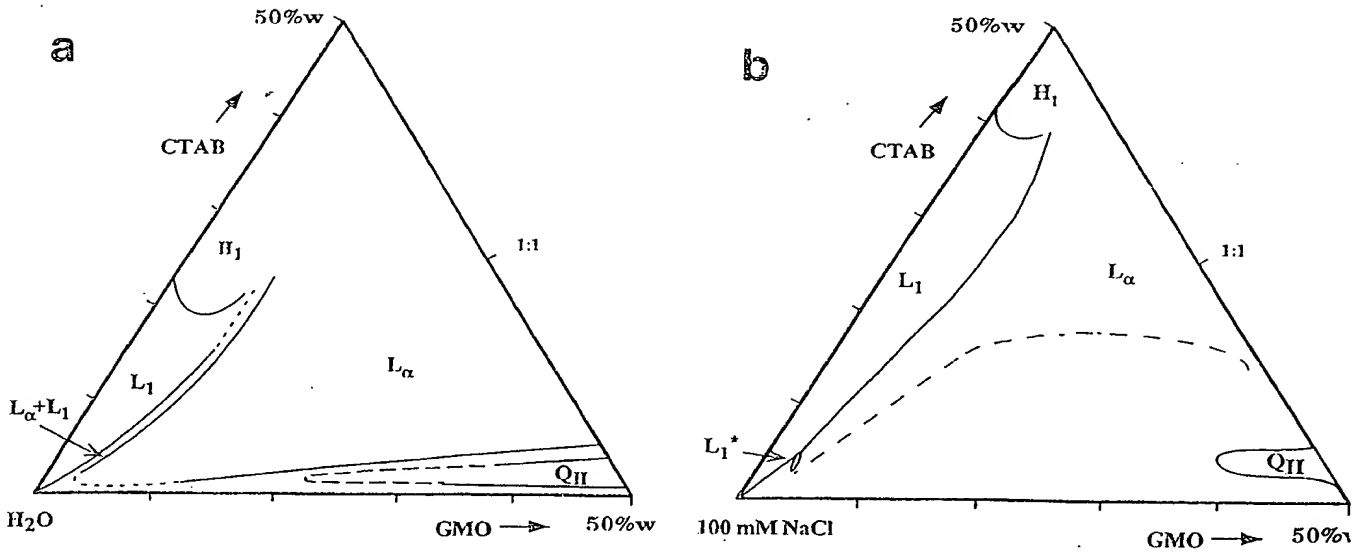

Fig. 9. Gibbs phase triangles for the systems C_{16}TAB/GMO/water and C_{16}TAB/GMO/0.100 M NaCl. The diagrams are similar gross features with those for the lecithins, Figs. 4 and 5. Note, however, the cubic phase Q_{II} which swells from about 40% of wa without surfactant (not shown) to 75% in water and 60% in brine. Reproduced and adapted with permission from [33]. Copyri 1998 American Chemical Society.

cubic phase. The lamellar phase, which is of prime interest here, is strikingly similar to that of EPC (Fig. 4), both with and without salt in the aqueous solvent. In particular, the narrow tongue that remains swollen in 0.100 M NaCl, also in the GMO system, seems to be stabilized by undulation forces, which are strengthened by a reduction of the rigidity of the bilayers from water-filled defects [33]. Just as in the phospholipid systems the formation of vesicles is facilitated when the bilayers become charged, and vesicles and open bilayer flakes with defects in the membranes are formed when salt is present. The perforated structures are solubilized without a clear two-phase region when more surfactant is added, forming lace-like structures and long thread-like or band-like micelles. In the absence of added salt the vesicles are without perforations, and no lace-like structures are found. The solubilization is more distinct with a two-phase region in the phase diagram (Fig. 9), but now both small globular micelles and long micelles formed as twisted ribbons are found together with defect free vesicles (Fig. 10) [33].

2.2.4. EPC/$C_xSO_4^-Na^+$/water or brine

The solubilization of sonicated EPC liposomes by anionic surfactants of the sodium dodecylsulfate (SDS) family has been investigated by turbidity a cTEM studies [34]. The solubilization processes a similar to those observed with cationic surfactant Without salt there is just a dissolution of the sm vesicles into mixed micelles, without any intermedia increase of the turbidity, whereas with 0.100 or 0.1 M NaCl growth occurs before the solubilization. Tl cTEM investigations reveal an interesting variatic of the morphology, strongly depending upon the a kyl chain length. The surfactant with the shorte chain (C_{10}) at 0.150 M salt gave a normal three-sta course: large vesicles and open bilayers dissolve int thread-like micelles, which then gradually shorten i the L_1 area. The behavior is very different with SD and the C_{14} homologue. Now the large intermediat vesicles have perforated or disrupted membrane and are found together with open perforated bilaye flakes. With a further increase of the surfactant cor centration, these structures turn directly into globu lar micelles without any thread-like micelles as inte mediates. Just as when C_{16}TAC in 0.100 M salt i used to solubilize EPC, it is not clear where the la mellar phase stops and the micellar phase starts. It i possible that the regions where small perforated disk are seen together with micelles (in the case of SDS) or lace-like structures and entangled threads (in th

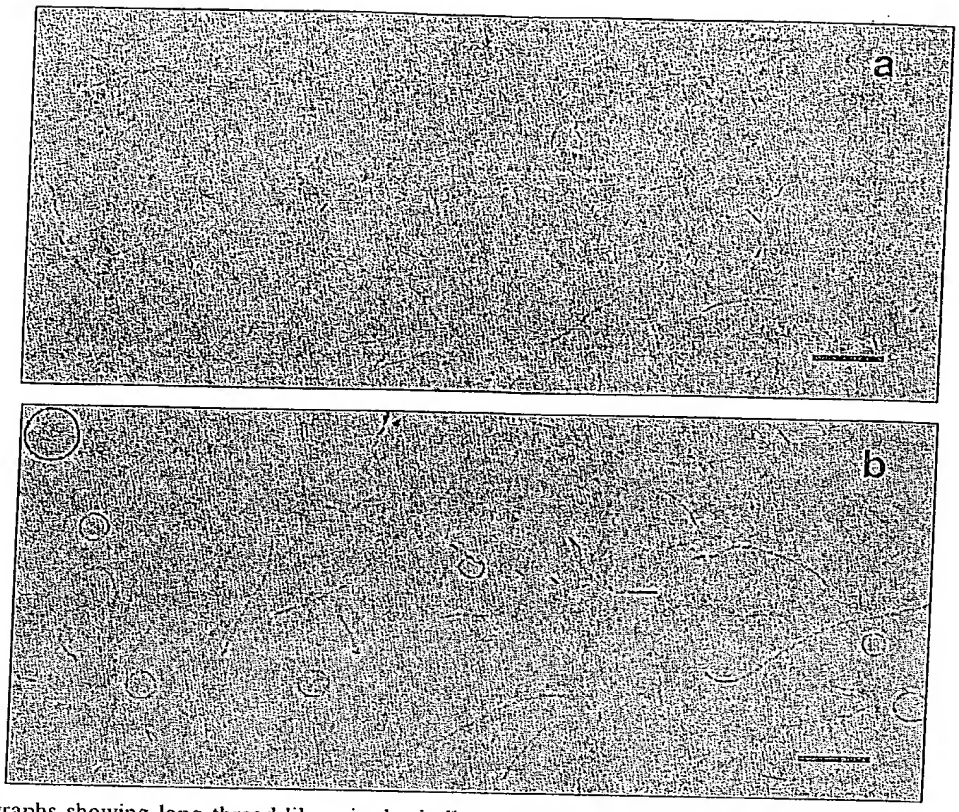

Fig. 10. cTEM micrographs showing long thread-like mixed micelles, apparently formed as twisted bands, in C_{16}TAB/GMO mixtures in 99.7% water. The weight fraction of surfactant to lipid was (a) 0.67 and (b) 0.47. In addition there are small micelles present in both, and in b also vesicles with intact bilayers. Reproduced with permission from [33]. Copyright 1998 American Chemical Society.

case of C_{16}TAC), belong to the two-phase area, although the solution appears as an L_1 phase, without birefringence or other indications of a lamellar phase. The lace-like structures and the perforated flakes would then be dispersed bilayers, and not equilibrium structures formed reversibly in the L_1 phase [34].

For SDS, when the salt concentration was reduced to 0.100 M, the perforated intermediates disappeared, and disk-like structures were observed, turning into globular micelles at higher surfactant concentrations. Thread-like micelles were only found in systems without salt, at high SDS concentrations, still with discoid structures appearing at lower surfactant concentration [34].

2.3. EPC or GMO/Na-cholate/water or brine

The interactions of bile salts with polar lipids are of particular interest because of the physiological significance of the bile salts. They effectively solubilize bilayer-forming lipids such as phospholipids and monoglycerides. A detailed phase diagram of lecithin and sodium cholate in water was published in 1966 by Small et al. [35,64], and molecular models of the structures of the mixed micelles and bilayers were proposed in 1967 [36,37]. These models have been very influential, and for a long time it was accepted as a fact that the mixed micelles were circular disk-like structures, comprising a bilayer of lecithins coated on the perimeter by bile salt molecules, exposing their hydrophilic surfaces to the surrounding aqueous solution, and their hydrophobic surfaces to the hydrophobic tails of the lipids. The long cylindrical micelles, which assembled into the hexagonal phase at high concentrations, were supposed to be built by stacking of discrete disk units. From static and dynamic light scattering results, Mazer et al. [38,39] proposed a modified model, retaining the disks, but now assuming that the bilayer contained

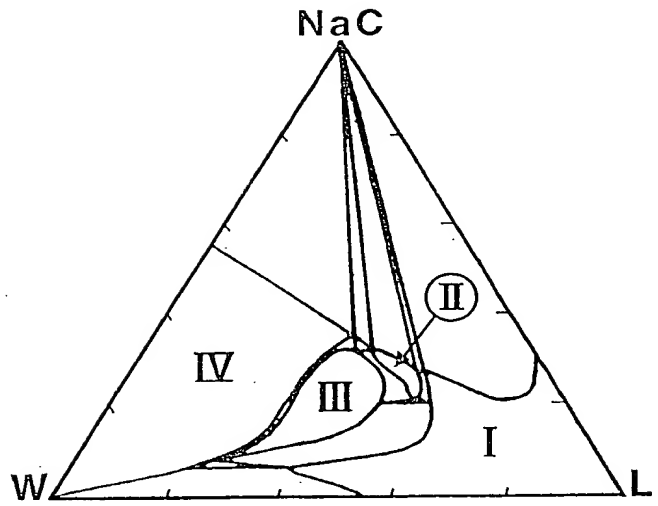

Fig. 11. The three-component phase diagram for sodium cholate/EPC/water as determined by Small et al. [35,64]. I to IV represent lamellar, bicontinuous cubic, hexagonal, and micellar phases, respectively. Note the three-phase triangle IV–III–I which guarantees that no hexagonal phase appears when the bile salt is added to the lecithins in excess water, but only lamellar phase and mixed micelles. Reproduced and adapted with permission from [35]. Copyright 1966 Elsevier Science.

some inserted bile salt molecules, possibly as hydrogen-bonded dimers.

Subsequently, results from small angle neutron scattering (SANS) were found to disagree with the disk-like micelle model, and indicated instead that rod-like structures were predominant [40,41]. A combination of careful static and dynamic light scattering measurements [42] and SANS experiments [43] showed that long flexible micelles were formed such structures were also directly imaged by cTEM [10,44]. NMR relaxation and diffusion studies of the hexagonal phase [45] had already shown that the lipid diffusion in the cylinders of the hexagonal phase was about as facile as in a neat liquid, and showed clearly that the cylinders had a continuous hydrophobic core that was liquid-like. This result refuted both the idea of the rod as a stack of disks, and in my mind also any model that prescribes a very precise molecular packing of the lipids and bile salts in the structures – we are dealing with mixed micelles not crystalline material. Anyway, now it seems to be settled that rod-like micelles are the important intermediates in the solubilization of lecithin bilayers by bile salts. However, by time-resolved SANS studies [46], it has recently been shown that the postulated [47,65] disk-like intermediates really are present as

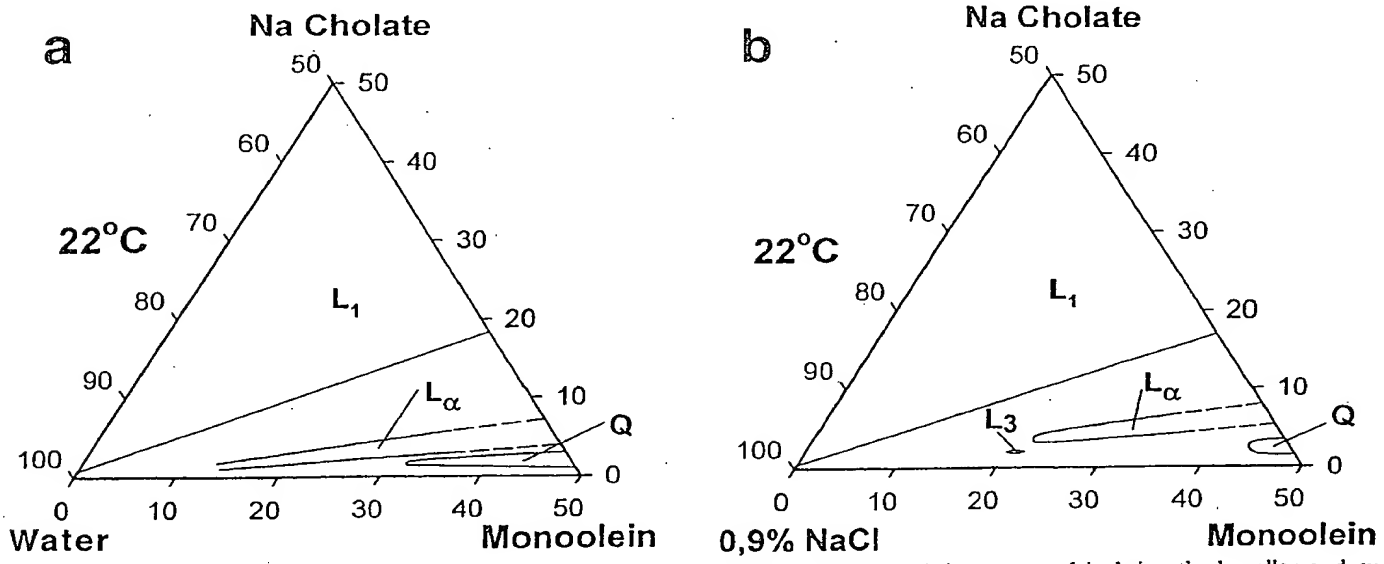

Fig. 12. The systems sodium cholate/GMO/water (a) and 0.9 wt% NaCl (b) at 22°C. Both in water and in brine the lamellar and cubic phases swell strongly, in water to a limit hard to determine exactly. Note the appearance of a L_3 phase, a so-called sponge phase or 'molten cubic' phase in the brine system, and that the micellar phase is very dominant in both systems, with almost the same solubility limit. Reproduced and adapted with permission from [48]. Copyright 1999 Academic Press.

short-lived transient species in the reversed process where vesicles are formed from mixed micelles by dilution (which due to the comparatively high solubility of the bile salt reduces the concentration of bile salt in the micelle, so that bilayers form).

Phase diagrams are presented in Figs. 11 and 12, for EPC/Na-cholate/water [35,64], and GMO/Na-cholate/water or brine (0.150 M NaCl) [48]. In all cases we see that the micellar L_1 phase is very prominent, extending to a molar ratio of lipid to cholate of about 2 for both lecithin and GMO. The bile salts are thus very effective in breaking bilayers and solubilizing polar lipids. The GMO bilayer can take up only 0.14 cholate molecules per lipid, whereas the EPC bilayers can accommodate up to 0.3 molecules.

As stressed above, a significant difference between GMO and egg lecithin is the presence of a cubic phase in the former in excess water, whereas the lamellar phase is the signature of the membrane-building lecithins. Only a small change of the spontaneous curvature is required to turn the cubic phase of GMO into a lamellar phase, and only about 0.07% bile salt is required in this case [48]. In the monoolein system both the cubic and the lamellar phases are strongly swollen in water, extending in narrow tongues towards the water corner in the phase diagram. In 0.150 M NaCl the swelling is weaker, and an island of L_3 phase is present between the tip of the lamellar phase and the aqueous corner. In all cases there are clear two- or multi-phase areas between the lamellar phase and the micellar phase. In the lecithin system both a hexagonal phase and at lower water content a cubic phase have been identified between the L_α phase and L_1. Neither of these

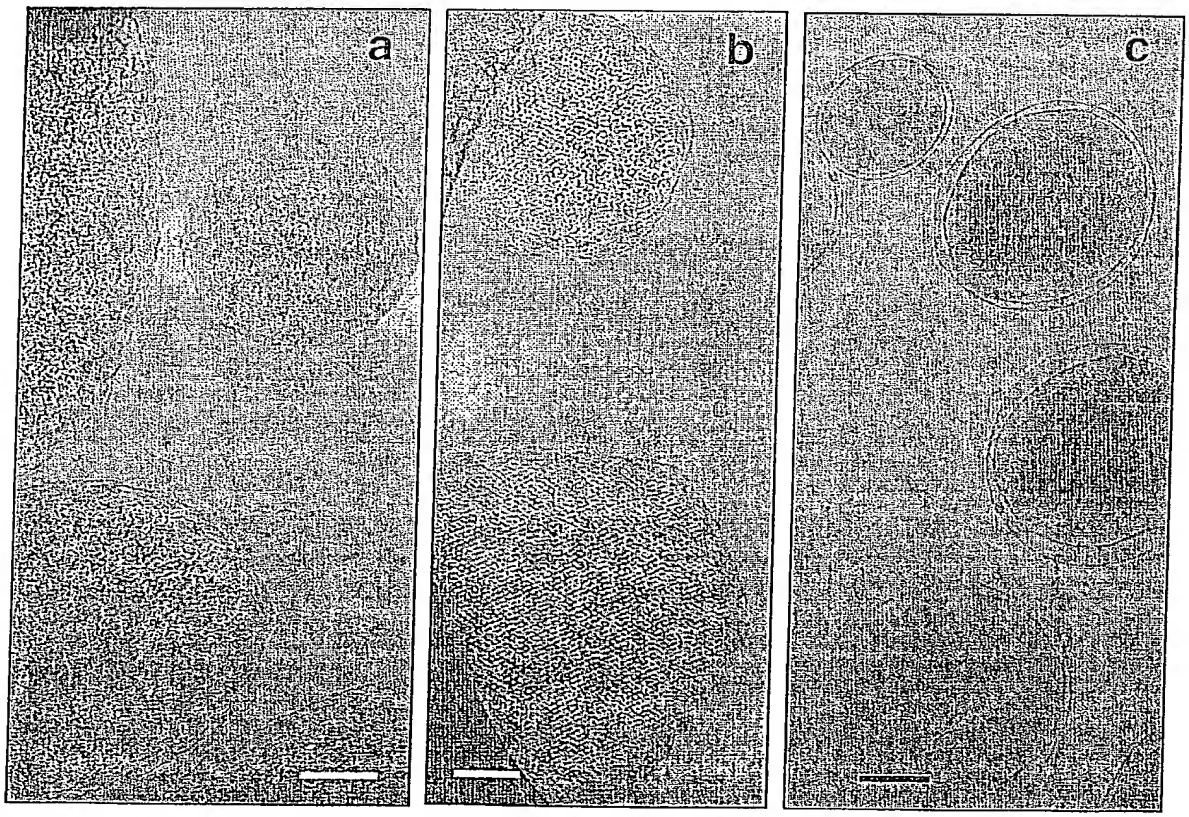

Fig. 13. (a, b) Particles observed in dispersions prepared by diluting the L_3 phase in excess brine (to 99% solvent). The particles in a have a non-periodic inner structure and may represent fragments of the L_3 phase, those in b have a periodic inner structure with hexagonal symmetry (and many defects) compatible with a cubic phase. (c) Passages and interconnections between the two membranes are found in this micrograph of double-walled liposomes formed on dilution of the lamellar phase in brine, sodium cholate/GMO = 0.15, 99% brine. Bar = 100 nm. Reproduced and adapted with permission from [48].

phases is in direct contact with the water corner, however. Instead a L_1, L_α, H_1 three-phase triangle blocks the contact. Addition of sodium cholate to a vesicle dispersion of the lamellar phase gives a rather typical three-stage course, where mixed rod-like micelles start to appear after saturation of the lamellar phase. The structures have been imaged by cTEM, and characterized in scattering studies, as discussed above. Interestingly, it was found that the solubilization was little dependent on the salt concentration in the solution [49]. Both R_{sol} and R_{sat} were practically the same in salt-free solutions and in 500 mM NaCl; it was observed, however, that growth of the vesicles prior to the formation of the mixed micelles occurred only with added salt, similar to other systems [31,34].

A dispersion of GMO in water or brine is not stable, but cubic phase particles quickly rejoin in domains of macroscopic dimensions. However, a little added charged surfactant increases the stability of the dispersion, at least so much that a cTEM investigation can be made, Fig. 13. Small dispersed particles of the cubic phase (cubosomes) have been found, and with salt present also particular dispersions of the L_3 phase. Dilution of the lamellar phase in excess brine, as in Fig. 13c, gives vesicles of interesting shape (without micelles). The vesicles are in general double-walled, with frequent interconnections between the two bilayers. This is understood as an influence from the nearby L_3 phase (which can be looked upon as a molten lattice of interpassages). When the lamellar phase is surrounded by excess aqueous solution, a substantial amount of the bile salt will escape from the membrane to the solution, and the composition of the bilayer will approach that of the L_3 phase. With more bile salt present, a broad coexistence region of bilayer structures, closed or open, and more or less globular micelles are found both in water and in brine. There are no thread-like micelles in this system. The mixed micelles seem to remain globular up to the L_1 saturation limit. This difference from the lecithin–bile system is in line with the absence of a hexagonal phase in the GMO system (Fig. 12). In the lecithin case, the phase diagram of Fig. 11 shows that the extrapolated L_1 border at lower water contents coincides with the hexagonal phase border, showing that this cholate/lecithin ratio favors formation of cylindrical aggregates.

3. Concluding summary and outlook

In this review I have discussed the solubilization o lipid bilayers by normal surfactants, and in particula the structures, intermediate or final, resulting fron the process. As far as possible I have tried to relat the particularities to phase relations. It is obviou that the understanding of these processes would b much improved if the phase behavior of the pertinen systems were mapped out in greater detail.

The so-called three-stage model for liposome sol ubilization has been generally successful, in particu lar for the non-ionic systems. But often a closer ex amination reveals perturbing features, related to th interactions between the structures, and often accom panied by a macroscopic phase separation. Mos noteworthy is the observation, first reported in [3 that on the solubilization of lecithin bilayers wit OG, the saturated bilayers equilibrate in a three phase region with two micelle phases, one dilut the other more concentrated and viscous, containin rod micelles.

There seem to be two main types of solubilizatio behavior. In the case of non-ionic surfactants, th lipid bilayers seem to reach 'saturation' becaus mixed micelles start to form in the aqueous solutio and the chemical potential of the surfactant sta almost constant after that point. This implies tha the composition of the bilayers also remains fixe during the dissolution. The phase behavior encoun tered with many charged surfactants is differen however. The lamellar phase seems to become dest bilized at a certain content of surfactant in the mem brane, and then disintegrates, forming mixed m celles, or a hexagonal phase, or an intermedia phase. In the phase diagram the stability limit is straight line, indicating the bilayer compositic where the spontaneous curvature of the monolay becomes too large for the planar structure to persis

In some systems, when salt is added, the two-pha area between L_1 and L_α becomes indistinct, and direct transition from a defect lamellar phase to micelle phase with unusual structure often seems occur. It is possible that the unusual structures reality are dispersed parts of the lamellar phase a two-phase area; a decisive test, which can also l applied to vesicles proposed to be thermodynam cally stable, is to investigate whether the structur

are equilibrium structures, in which case making and reversing a change of conditions should result in a reversible morphological change within the observation time after a change of conditions.

The defective intermediates, i.e. perforated vesicles and similar structures, are not entirely restricted to charged systems. There are suggestions, for example in [3], that a perforated lamellar phase is present in the EPC/OG/water system. Perforated vesicles, and a lamellar phase swelling in a narrow tongue to more than 97% of water, were also observed in a more unusual system, diglyceroldecanoate/glyceroldecanoate/water [50].

Bile salts are a special class of surfactants, and their behavior with polar lipids has been examined in great detail. The bile salts have a very good ability to solubilize polar lipids in mixed micelles, and seem to break down the bilayer already at comparatively low additions. Originally, disk-like mixed micelles were conjectured, with polar membrane lipids building the disk, and the bile salts covering the hydrophobic rim, but later work has shown that flexible cylinders are the dominant intermediates also in these systems, even if the disk-like structures have been re-established as transient intermediates in the transformation from mixed micelles to vesicles.

Disk-like intermediates – be they equilibrated micelles or bilayer fragments – have been established in many systems, with both non-ionic and ionic surfactants. Usually the disks are irregular in shape, but in some systems very planar and nicely circular disks have been found. An example was given in [51] where the effect of PEG lipids on DSPC and EPC liposomes was investigated. With about 10 mol% PEG lipid, circular and planar disks were obtained in both systems, instead of closed vesicles, when the lipid mixture of the bilayers had been fortified with 40 mol% of cholesterol, whereas irregular bilayer flakes were formed without cholesterol in the lipid mixture. The type of liposome membrane investigated in this example was devised for practical use (drug delivery), and is typically surrounded by hydrophilic polymer chains (such as PEG) for stabilization, anchored in the bilayer by covalent attachment to a polar lipid. Furthermore, they often have a high cholesterol content in the lipid mixtures, which improves the strength of the membrane and reduces leakage of entrapped hydrophilic substances, and also makes the bilayers more resistant to solubilization by surfactants [52,53]. The investigation of the stability of such more complex bilayer membranes has only begun. It can be anticipated that the rigid and mechanically tough membranes that are preferred for drug delivery applications will often give disk-like structures on disintegration by surfactants, but be harder to break down, since they withstand higher surfactant concentrations and solubilize slowly.

An important question is the effect on bilayer membranes of surfactants that are biologically relevant, such as lysolecithins and salts of fatty acids [54]. Except for systems with bile salts, such work has not been reviewed here, mainly because no systematic studies of the phase behavior seem to have been made as yet. Such investigations are needed. There are other topics that could have been given more attention in the review, such as studies of how variations of surfactant headgroup (charge [55], PEG size [56]), or the bilayer composition [57], influence the solubilization process. I have also chosen not to include studies of solubilization of 'synthetic' bilayer-forming surfactants, such as the extensive studies of the SDS–didodecyldimethylammonium bromide–water system [58,59] or the studies of dimer surfactants [60].

Theoretical treatments of the vesicle to mixed micelle transformation have not been covered here. Some simple models based on the differences in spontaneous curvature between surfactant and lipid have for example treated the transition from isolated bilayers to cylindrical micelles [61,62] and also discussed cylinder or disk issue [63].

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SALES SPECIFICATION

FOR		
EASTMAN® Vitamin E TPGS, NF Grade	**SPECIFICATION NO.**	19947-1
	NUMBER OF PAGES	2
	EFFECTIVE DATE	June 20, 2001

A. GENERAL

A-1. This Specification has been revised to reflect current nomenclature and format.

A-2. This specification describes a NF Grade of Eastman Vitamin E that is a low-melting, waxy, pale-yellow solid, and is in a water-soluble form. It is labeled to contain 260 milligrams per gram of d-alpha tocopherol. One (1) mg d-alpha tocopherol equals 1.49 International Units. This product shall meet all requirements listed below. It shall also conform to all requirements of the U. S. Pharmacopeia/National Formulary.

A-3. The final product shall have rabbinical certification.

A-4. Eastman's Material Safety Data Sheet outlines the safety precautions for EASTMAN® Vitamin E TPGS, NF Grade.

B. PROPERTIES AND SPECIFICATIONS

B-1.	Appearance	Waxy solid, white to light brown. Practically Free from particulate matter
B-2.	Potency	260 to 300 mg/g of d-alpha tocopherol per gram (after saponification of the ester)
B-3.	Acid Value	Not more than 1.5
B-4.	Color (Gardner Scale)	Not more than 10
B-5.	Heavy Metals (as Lead)	Not more than 10 ppm
B-6.	Specific Rotation (alpha) D_{25}	Not less than +24°

B-7	Solubility in Water	20% Minimum (Clear Solution)
B-8	Solvent contents	
	-Ethanol	Not more than 50 ppm
	-Methyl Ethyl Ketone	Not more than 10 ppm
	-Toluene	Not more than 10 ppm
B-9	Identification (IR Spectrum)	Corresponds to reference
B-10	Free Tocopherol	Not more than 1.5%

English Translation of WO 92/03122

International patent application

Preparation for the application of agents in mini-droplets

The present invention relates to a novel type of preparations suitable for the application of different agents in the form of a minuscule droplet or, in particular, a vesicle consisting of one or a few membrane-like amphiphile assemblies. These can mediate the transport of agents into and through a series of natural permeability barriers or through the constrictions in such barriers; for example, through intact skin or similar organs. The invention further relates to a procedure for the large-scale production of such carriers. As a special example, non-invasive application of antidiabetics is described for the case of insulin.

The application of various agents is often hampered by the presence of barriers with a low permeability to such agents. Owing to skin impermeability, for example, many common therapeutic agents must be applied per os or parenterally (i.v., i.m., i.p.). Intrapulmonary and intranasal applications of aerosols, the use of rectal formulations, gels for mucous applications, or use of occular formulations are only practicable in certain areas and not for all types of drugs. The transport of different agents into plant tissues is subject to even more severe constraints due to the high permeability barrier of the cuticular wax layers.

Noninvasive drug application through permeability barriers thus would be advantageous in many cases. In humans and animals one would expect such a percutaneous application of agents to protect the agents from degradation in the gastrointestinal tract; modified drug distribution could possibly also be achieved. Such drug application, moreover, would influence the pharmacokinetics of the agent molecules and permit simple as well as multiple noninvasive therapy. (Karzel K., Liedtke, R.K. (1989) Arzneim. Forsch./Drug Res. 39, 1487-1491). In the case of plants, improved penetration

into or through the cuticle could reduce the drug concentration required for a given application and thus significantly diminish pollution problems (Price, C.E. (1981) In: The plant cuticle (D.F. Cutler, K.L. Alvin, C.E. Price, Edits.), Academic, New York, pp. 237-252).

There are many reports on different attempts to increase the permeability of intact skin by suitable manipulations (cf. Karzel und Liedtke, op. cit.). Jet injection (Siddiqui & Chien (1987) Crit. Rev. Ther. Drug. Carrier. Syst. 3, 195-208.), the use of electric fields (Burnette & Ongpipattanakul (1987) J. Pharm. Sci. 76, 765-773) or chemical penetration enhancers, such as solvents and surfactants, are particularly worth mentioning. A long list of additives which have been used to enhance the penetration of one particular water soluble agent (Nolaxon) into skin, for example, is given in the work by Aungst et al. (1986, Int. J. Pharm. 33, 225-234). This list encompasses nonionic substances (including long-chain alcohols, surfactants, zwitterionic phospholipids, etc.), anionics (most notably fatty acids), cationic long-chain amines, sulfoxides as well as different amino-derivatives; amphotheric glycinates and betaines are also mentioned. Despite all this, the problem of agent penetration into skin has as yet not at all - or not satisfactorily - been solved.

A survey of procedures used for increasing the penetration of agents through a plant cuticle is given in the work by Price (1981, op.cit.). To date it has been common to simply add chemical penetration enhancers to the mixture of agent and other molecules; applications to human skin were the only case in which additives were sometimes applied in advance, in the form of an organic solution. The reason for this application form was the current concept for the action of penetration enhancers: to date one has studi d, discussed, and believed

that, in general, any facilitated agent penetration is a consequence of skin fluidization, on the one hand (Golden et al., (1987) J. Pharm. Sci. 76, 25-28). (This phenomenon is normally associated with a destruction of the skin surface and of its protective shield and thus is undesired.) On the other hand, it has been shown that some agents can permeate through skin in the form of low-molecular weight complexes with added molecules (Green et al., (1988) Int. J. Pharm. 48, 103-111).

Methods deviating from the ones already described have brought little improvement to date. The use of lipoidal carriers, the liposomes, on intact skin, which has been theoretically discussed by several authors, was mainly aimed at modifying the agent's pharmacokinetics (Patel, Bioch. Soc. Trans., 609th Meeting, 13, 513-517, 1985, Mezei, M. Top. Pharm. Sci. (Proc. 45th Int. Congr. Pharm. Sci.F.I.P.) 345-58 Elsevier, Amsterdam, 1985). Thus far, all proposal of this kind, moreover, involved the use of standard lipid vesicles (liposomes) which cannot penetrate the skin at all or permeate through the skin very inefficiently, as is shown in this patent application. Patent applications nos. JP 61/271204 A2 [86/271204] refer to a related use of liposomes in which hydrochinonglucosidal is employed to improve the stability of the agent.

Hitherto available preparations for percutaneous use have mostly been applied under occlusion; in the case of liposomal preparations, this was even a general rule. The corresponding preparations only contained small or lipophilic substances, as well as a limited number of skin-fluidizing additives. Correspondingly, they afforded only partial control over the pharmacokinetic properties of final preparations. In an attempt to improve this situation a proposal was made (WO 87/1938 A1) to use drug-carrying lipid vesicles in combination with a gelatinizing agent as a transdermal patch. This has

prolonged drug action but has not increased the skin-penetration capability of the drug itself. Through massive use of penetration enhancers (polyethylene glycol and fatty acids) and of lipid vesicles, Gesztes und Mezei (1988, Anesth. Analg. 67, 1079-1081) have succeeded in inducing local analgesia with lidocaine-containing carriers; however, the overall effectiveness of the drug in this preparation was relatively low and its effects were only observed several hours after the beginning of an occlusive application.

By a specially designed formulation we have succeeded in obtaining results which were dramatically better than those of Gesztes and Mezei. Our carrier formulations consisted of filtered lipid vesicles (liposomes) which also contained some detergents, with a declared optimum lipid/surfactant content of 1-40/1, in practice mainly around 4/1.

These results provided a basis for German patent application P 40 26 834.9-41 which also refers to German patent application P 40 26 833.0-43; the latter deals with the problem of liposome fabrication.

Since then, we have unexpectedly discovered that certain criteria, described in this application, may be formulated for the qualification of drug carriers as suitable for the penetration into and through a permeability barrier. The main requirement of such a drug carrier - which in the following is called a transfersome - is that it is sufficiently elastic to penetrate through the constrictions in a barrier, such as skin. In the case of transfersomes consisting of phosphatidylcholine and sodium cholate this condition is fulfilled when the edge tension of a carrier is below 10 Piconewton; similar values are also likely to pertain to other, related systems. Carriers which are capable of creating a gradient after an application are particularly

useful; this is due to the fact that they have a spontaneous tendency for penetration through permeability barriers.

It is, therefore, an object of the present invention to specify the properties of novel preparations which are suitable for the mediation of rapid transport of diverse agents and other substances through permeability barriers and constrictions.

A further object of this invention is to introduce a new class of carrier preparations for the transport of drugs through human, animal or plant skin, which result in a characteristic improved availability of the agent molecules at the target site.

It is yet another object of this invention to prepare formulations for non-invasive application of antidiabetics, most notably of insulin; these should ensure an improved, therapeutically sufficient, and reproducible form of drug application.

A further object of this invention is to provide procedures for the production of such preparations.

These objects have been accomplished through the features of the independent claims.

Advantageous embodiments are mentioned in the subclaims.

The transfersomes according to this invention differ from the liposomes hitherto described for topical application and from other related carriers in at least three basic features. Firstly, they can consist of an arbitrary amphiphile, including oils. Secondly, they can be made in arbitrary fashion: their penetration capacity does not depend on the

manufacturing procedure. Thirdly, the penetration capability of the previously described liposomes optimized for applications on skin (cf. patent application P 40 26 834.9-41) was based on the use of a carrier composition with an optimal lipid/surfactant ratio in the range of L/S=1-40/1. However, a transfersome must mainly have an optimal elasticity, which ensures a sufficiently high permeation capability of such a carrier. If this basic requirement is fulfilled by the addition of edge-active substances to a basic transfersome component, the necessary total amount of the edge-active substance can correspond to L/S values below 1/500 (in the case of classical surfactants below 1/50 to 1/100). The range of concentrations suitable for making transfersomes is thus by several thousand per cent higher than previously believed.

Transfersomes also differ from micellar carrier formulations in at least two basic features. Firstly, a transfersome is, as a rule, far bigger than a micelle; consequently, it also obeys different diffusion laws. Secondly, and more importantly, a transfersome typically contains a water-filled central core (the inner lumen of a vesicle). Nearly all water soluble substances can be incorporated in the core of a transfersome and thus transported across a permeability barrier. Transfersomes are suitable for transporting amphiphilic and lipophilic substances.

If simple carriers are not sufficiently deformable and their permeation capacity must be achieved by using certain edge-active additives, the concentration of the latter is then preferably in the range between 0.1 and 99 % of the quantity which would be required for carrier solubilization. Frequently, the optimum - depending on the purpose and the drug used - is located in the range between 1 and 80 %, most frequently between 10 and 60 % of the solubilization dose; the

concentration range between 20 and 50 mol-% is the most preferred dose.

Our novel transfersomes can mediate transport of agents through essentially all permeability barriers and are suitable, for example, for percutaneous (dermal) applications of medical agents. Transfersomes can carry water- or fat-soluble agents to various depths at the application site, depending on the transfersomal composition, application dose, and form. Special properties which cause a carrier to behave as a transfersome can be realized for phospholipid vesicles as well as for other types of amphiphile aggregates.

In this application it is shown for the first time that by means of suitably formulated transfersomes, a major proportion of the drugs applied can be introduced not only into a permeability barrier, such as skin, but, moreover, can be transported into the deeper tissues where they become systemically active. Transfersomes can carry polypeptides, for example, through intact skin at an effectiveness which is a 1,000 times higher than was previously possible when using structureless penetration enhancers. Transfersomally formulated substances can reach nearly 100 % of the corresponding biological or therapeutical maximum efficacy after applications on human skin. Similar effects, to date, have only been achievable by using an injection needle.

In the course of this study, it has surprisingly been found that through use of such novel drug carriers, antidiabetics can be brought into the blood through intact skin without the necessity of auxiliary measures such as an injection. After a dermal application of insulin applied in the form of transfersomes, more than 50 % and often more than 90 % of the applied drug dose are routinely found in the destined organs of the body. Insulin-containing, dermally applied

transfersomes can thus successfully replace injections of insulin solutions.

The present invention, consequently, opens up a way for simple, noninvasive and completely painless therapy of type II diabetes: transfersomes can be used alone or in combination with an arbitrary dosing means for non-problematic therapy of acute and/or chronical diabetes.

Carriers according to this invention can consist of one or several components. Most commonly, a mixture of basic substances, one or several edge-active substances and agents is used. Lipids and other amphiphiles are best suited basic substances; surfactants or suitable solvents are the best choice from the point of view of edge-active substances. All these can be mixed with agents in certain proportions depending both on the choice of the starting substances and on their absolute concentrations. It is possible that one or several preparation components are only made edge-active by subsequent chemical or biochemical modification of a preparation (ex tempore and/or in situ).

Transfersomes thus offer an elegant, uniform and generally useful means of transport across permeability barriers for diverse agents. These newly developed carriers are perfectly suited for use in human and animal medicine, dermatology, cosmetics, biology, biotechnology, agrotechnology and other fields.

A transfersome according to this invention comprises any carrier with a special capability to get or diffuse into or through a permeability barrier under the effect of a gradient and by so doing to transport material between the application and destination sites.

A (drug) carrier of this type preferably corresponds to a molecular homo- or hetero-aggregate or to a polymer. The carrier aggregate, according to this invention, consists of a few or many, identical or different molecules; these form a physico-chemical, physical, thermodynamical and, quite frequently, functional unity. Some examples of corresponding aggregates are micelles, disk-micelles, oil-droplets (nanoemulsions), nanoparticles, vesicles or 'particulate emulsions'; parts of an aggregate can also be held together by (a) non-covalent force(s). The optimal carrier size is also a function of the barrier properties. Furthermore, it is influenced by the polarity (hydrophilicity), mobility (dynamics), and charge density as well as the elasticity of an carrier (surface). Advantageous sizes of transfersomes are in the range of 10 nm to 10,000 nm.

For dermal applications, for example, preferably particles or vesicles with a diameter of the order of 100-10,000 nm, frequently in the range of 100 to 400 nm, and most frequently with sizes between 100 and 200 nm are used as carriers.

For the use in plants, relatively small carriers, depending on the details of each individual application, should be used, most frequently with diameters below 500 nm.

DEFINITIONS

LIPIDS

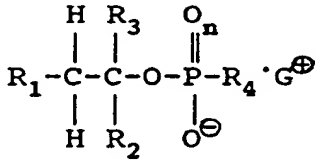
A lipid in the sense of this invention is any substance with characteristics similar to those of fats or fatty materials. As a rule, molecules of this type possess an extended apolar

region (chain, X) and, in the majority of cases, also a water-soluble, polar, hydrophilic group, the so-called head-group (Y). The basic structural formula 1 for such substances reads

$$X - Y_n \qquad (1)$$

where n is greater or equal zero. Lipids with n=0 are called apolar lipids; those with n >= 1 are polar lipids. In this context, all amphiphiles, such as glycerides, glycerophospholipids, glycerophosphinolipids, glycerophosphonolipids, sulfolipids, sphingolipids, isoprenoidlipids, steroids, sterines or sterols and lipids containing carbohydrate residues, can simply be referred to as lipids.

A phospholipid, for example, is any compound of formula 2



(2)

In this formula, n and R_4 have the same significance as in formula 8 except that R_1 and R_2 cannot be hydrogen, an OH-group or a short chain alkyl residue; R3 is a hydrogen atom or an OH-group, in the majority of cases. In addition, R_4 can be a short chain alkyl group substituted by three short chain alkylammonium residues, e.g. trimethylammonium, or an amino-substituted short chain alkyl, e.g. 2-trimethylammonioethyl (cholinyl).

A lipid is preferably any substance according to formula 2, in which n=1, R_1 and R_2 is hydroxyacyl, R_3 is a hydrogen atom and R_4 is a 2-trimethylammonioethyl (the last compound corresponding to the phosphatidylcholine headgroup), 2-dimethylammonioethyl, 2-methylammonioethyl or 2-aminoethyl (corresponding to

a phosphatidylethanolamine headgroup).

A lipid of this kind is, for example, phosphatidylcholine from natural sources, in the old nomenclature also called lecithin. This can be obtained, for example, from eggs (then being rich in arachidic acid), soy-bean (rich in C-18 chains), coconuts (rich in saturated chains), olives (rich in monounsaturated chains), saffron, safflower and sunflowers (rich in n-6 linolenic acid), linseed (rich in n-3 linolenic acid), from whale-oil (rich in monounsaturated n-3 chains), from Nachtkerze or primrose (rich in n-3 chains), etc. Preferred natural phospsphatidylethanolamines (in the old nomenclature also called cephalins), frequently stem from egg or soy-beans.

Further preferred lipids are synthetic phosphatidylcholines (R_4 in formula 2 corresponding to 2-trimethylammonioethyl), synthetic phosphatidylethanolamines (R_4 being identical to 2-aminoethyl), synthetic phosphatidic acids (R_4 being a proton) or their esters (R_4 corresponding e.g. to a short chain alkyl, such as methyl or ethyl), synthetic phosphatidylserines (R_4 corresponding to an L- or D-serine), or synthetic phosphatidyl(poly)alcohols, such as phosphatidylglycerol (R_4 being identical to L-or D-glycerol). In this case, R_1 and R_2 are identical acyloxy residues such as lauroyl, oleoyl, linoyl, linoleoyl or arachinoyl, e.g. dilauroyl-, dimyristoyl-, dipalmitoyl-, distearoyl-, diarachinoyl-, dioleoyl-, dilinoyl-, dilinoleoyl-, or diarachinoylphosphatidylcholine or -ethanolamine, or different acyl residues, e.g. R_1 = palmitoyl and R_4 = oleoyl, e.g. 1-palmitoyl-2-oleoyl-3-glycerophosphocholine; or different hydroxyacyl residues, e.g. R_1 = hydroxypalmitoyl and R_4 = hydroxyoleoyl; or mixtures thereof, e.g. R_1 = hydroxypalmitoyl and R_4 = oleoyl etc. R_1 can also signify an alkenyl and R_2 identical hydroxyalkyl residues, such as tetradecylhydroxy or hexadecylhydroxy, e.g.

in ditetradecyl- or dihexadecylphosphatidylcholine or -ethanolamine, R_1 can be an alkenyl and R_2 a hydroxyacyl, e.g. a plasmalogen (R_4 = trimethylammonioethyl), or R_1 can be an acyl, e.g. myristoyl, or palmitoyl, and R_2 a hydroxy, e.g. in natural or synthetic lysophosphatidylcholines or lysophosphatidylglyceroles or lysophosphatidylethanolamines, e.g. 1-myristoyl- or 1-palmitoyllysophosphatidylcholine or -phosphatidylethanolamine; R_3 is frequently hydrogen.

A convenient lipid according to this invention is also a lipid of the basic formula 2, in which n=1, R_1 is an alkenyl residue, R_2 is an acylamido residue, R_3 is a hydrogen atom and R_4 is 2-trimethylammonioethyl (choline residue). A lipid of this kind is known under the term sphingomyeline.

Furthermore, suitable lipids are analogs of lysophosphatidylcholine, such as 1-lauroyl-1,3-propandiol-3-phosphorylcholine, monoglycerides, such as monoolein or monomyristin, a cerebroside, a ganglioside or a glyceride which contain no free or esterified phosphoryl- or phosphono group or a phosphino group in the position 3. One example of such glyceride is diacylglyceride or 1-alkenyl-1-hydroxy-2-acylglyceride with arbitrary acyl or alkenyl groups, the 3-hydroxy group in these then being ether-bonded to one of the mentioned carbohydrate residues, such as a galactosyl residue, for example in monogalactosylglycerol.

Lipids with desired head or chain group properties can also be prepared biochemically, using e.g. phospholipases (such as phospholipase A1, A2, B, C, and especially D), desaturases, elongases, acyl-transferases, etc., starting with any natural or synthetic precursor.

Suitable lipids, furthermore, are all lipids found in

biological membranes and extractable with suitable apolar organic solvents, such as chloroform. In addition to the lipids already mentioned, this group of lipids also encompasses steroids, such as oestradiols, or sterines, such as cholesterin, beta-sitosterine, desmosterine, 7-keto-cholesterin or beta-cholestanol, fat-soluble vitamins, such as retinoids, vitamins, such as vitamin A1 or A2, vitamin E, vitamin K, such as vitamin K1 or K2, or vitamin D1 or D3, etc.

EDGE ACTIVE SUBSTANCES

An edge active substance according to this application is any substance which is capable of inducing or increasing the carrier system's capacity to form edges, protrusions or relatively strongly curved surfaces; this property also manifests itself in the capability to induce pores in lipid structures, such as membranes, or even provoke a solubilization (lysis) in the higher concentrations ranges. More strictly speaking, all such substances are considered edge-active which exhibit a tendency to accumulate at or near the edges between the polar and apolar parts of molecules and/or near or at the edges between the polar and apolar parts of the supramolecular aggregates, thereby lowering the free energy for the formation of edges and/or strongly curved surfaces. All surfactants and many solvents as well as asymmetric, and thus amphiphatic, molecules or polymers, such as many oligo- and polycarbohydrates, oligo- and polypeptides, oligo- and polynucleotides or their derivatives also belong to this category.

The edge activity of the used 'solvents', surfactants, lipids, or agents depends on the effective relative hydrophilicity or hydrophobicity of each molecule, and can also be modified by the choice of further system components and boundary conditions in the system (temperature, salt content, pH value,

etc.). Functional groups, such as double bonds in the hydrophobic part of molecules, which lower the hydrophobicity of this molecular region, increase edge activity; elongation or space-demanding substituents in the hydrophobic molecular parts, e.g. in the aromatic part, lower the edge activity of a substance. Charged or strongly polar groups in the headgroup normally increase the edge activity provided that the hydrophobic molecular part has remained the same. Direct connections between the lipophilic and/or amphiphilic system components have the reverse effect.

Solvents which are to some extent edge active only in certain concentration ranges encompass simple, especially short chain, alcohols, such as methanol, ethanol, n-propanol, 2-propen-1-ol (allylalcohol), n-butanol, 2-buten-1-ol, n-pentanol (amylalcohol), n-hexanol, n-heptanol, n-octanol and n-decanol; furthermore, iso-propanol, iso-butanol or iso-pentanol. Higher alcohols are even more potent, for example, ethandiol (ethylene glycol), 1,2-propane diol (propylene glycol), 1,3-propane diol, 1,3-butane diol, 2,3-butane diol, propane triol (glycerol), 2-butene-1,4-diol, 1,2,4-butane triol, 1,3,4-butane triol, 1,2,3-butane triol, butane tetraol (erythritol), 2,2-bis(hydroxymethyl)1,3-propane diol (pentaerythritol), 2,4-pentadiol and other pentadiols or pentendiols, 1,2,5-pentantriol and other pentantriols or pententriols, pentantetraol, 1,2,6-hexane triol and other hexane triols, hexane tetraol and -pentaol, heptane diol, - triol, -tetraol, -pentaol and -hexaol, 1,4-butane diol- diglycidyl-ether, etc. Short-chain, di-, tri-, tetra-, penta- and hexa-oxyethylene glycols and -ethylene glycols are also suitable for the present purpose as well as cyclic alcohols, such as benzylalcohol, cyclopentanol, cyclohexanol, 3-, 4-, 5-cyclohexanol, cyclohexylalcohol, aryl-alcohols, such as phenyl-ethanol, etc.

Edge active solvents which can be used according to this invention include, furthermore, short-chain acyl-, alkyl-, alkenyl, hydroxyacyl-, alkenyloxy- as well as aryl derivatives of different acids and bases, such as acetic acid, formic acid, propionic acid, butenoic acid, pentenoic acid, etc. of many amino acids, benzoic acid, phosphoric- and sulphuric acid, of ammonia, purine, pyrimidine, etc., provided that they do not impair the chemical integrity of the carriers and the agent molecules to an inacceptable extent.

A nonionic edge active substance is any material which contains at least one, and in the majority of cases several, strongly hydrophilic groups and at least one, sometimes also several relatively hydrophobic, water insoluble residues. 'Nonionic' edge active substances can be zwitterionic or truly non-ionic.

Free of any charge and edge active are e.g. the lipoidal substances of the basic formula 3

$$R_1 - ((X_i - Y_j)_k - Z_l)_m - R_2 \qquad (3)$$

in which X, Y and Z are different polar (hydrophilic) or apolar (hydrophobic) groups, which confer an amphiphatic character to the whole molecule. Z ist mainly a water soluble residue and i, j, k, l and m are greater or equal zero. R_1 and R_2 are two arbitrary residues; the first is mostly polar or very short; the second apolar.

The residues R_2 or X in such lipids often represent an acyl-, alkyl-, alkenyl-, hydroxyalkyl-, hydroxyalkenyl- or hydroxyacyl-chain with 8-24 carbon atoms. Very frequently, n-hexyl, n-heptyl, n-octyl, n-nonyl, n-decyl, n-undecyl, n-dodecyl, n-tetradecyl or n-tetradecenoyl, n-hexadecyl, n-

hexadecenoyl, n-octadecyl, n-octadecenoyl and n-octadecendienyl, n-octadecentrienyl, etc. are used.

Sorbitol is one possible example of residue Z. (X_i - Y_j) can be a polyene, polyoxyalkene, such as polyoxyethylene, polyalcohol, such as polyglycol, or polyether. (X_i - Y_j) mainly contain 1-20 and very frequently 2-10 units, e.g. in ethylene glycol, di- and triglycol (oligoglycol) or polyethylene glycol.

In simple substances according to formula 3, the residue R_1 or R_2 is frequently an alkyl-, alkenyl-, hydroxyalkyl-, alkenylhydroxy- or hydroxyacyl-chain with 1-24 carbon atoms. Very suitable are substances such as n-dodecyl (lauryl-ether), n-tetradecyl (myristoyl-ether), n-pentadecyl (cetyl-ether), n-hexadecyl (palmitoyl-ether), n-octadecyl (stearoyl-ether), n-tetradecenoyl (myristoleoyl-ether), n-hexadecenoyl (palmitoleoyl-ether) or n-octadecenoyl (oleoyl-ether). Owing to their good availability, the following substances are, amongst others, frequently used: 4-lauryl-ether (Brij 30), 9-lauryl-ether, 10-lauryl-ether, 23-lauryl-ether (Brij 35), 2-cetyl-ether (Brij 52), 10-cetyl-ether (Brij 56), 20-cetyl-ether (Brij 58), 2-stearyl-ether (Brij 72), 10-stearyl-ether (Brij 76), 20-stearyl-ether (Brij 78), 21-stearyl-ether (Brij 721), 2-oleoyl-ether (Brij 92), 10-oleoyl-ether (Brij 96) and 20-oleoyl-ether (Brij 78), the increasing number in their names indicating an increasing headgroup length. Suitable substances of this class are marketed under the names GENAPOL, THESIT and LUBROL.

Amongst the most common nonionic surfactants of the ether-type which are suitable for the present purpose are the substances of the Myrj trademark, such as polyoxyethylene(8)-stearate (Myrj45), polyoxyethylene(20)-stearate (Myrj49), polyoxy-.

ethylene(30)-stearate (Myrj51), polyoxyethylene(40)-stearate (Myrj52), polyoxyethylene(50)-stearate (Myrj53), polyoxyethylene(100)-stearate (Myrj59), etc. Further products of these classes are sold under the trademark Cirrasol ALN; common polyoxyethylene-alkylamides are e.g. surfactants of the trademark Atplus.

Another important special form of the nonionic edge active substance according to basic formula 3 most frequently contains a hydroxyl group in the position of residue R_1 and a hydrogen atom in the position of residue R_2, by and large. Residues X and Z are frequently an alkoxy- or alkenoxy-, in principle also a hydroxyalkyl-, hydroxyalkenyl- or hydroxyacyl-chain with 4-100 carbon atoms. Residue Y, too, is frequently an alkoxy-, alkenoxy-, hydroxyalkyl-, hydroxyalkenyl- or hydroxyacyl-chain but one which is often branched and carries one methyl-or ethyl-side chain. Perhaps the most widely used edge active substances of this class are the surfactants which are marketed unter the trademark "Pluronic".

Further, very commonly used special forms of non-ionic edge active substances are sold under the trademark "TWEEN". The cyclic part of this substance class is frequently a sorbitol ring. Residues R_1, R_2, R_3 and R_4 are frequently of the alkoxy- or alkenoxy-, and even more commonly of the polyene-, polyoxyalkene-, such as polyoxyethylene-, polyalcohol-, such as polyglycol-, or polyether type. Some of these chains can be apolar, corresponding to e.g. an acyl-, alkyl-, alkenyl-, hydroxyalkyl-, hydroxyalkenyl- or hydroxyacyl-chain with 8-24 carbon atoms. If none of residues R_1, R_2, R_3 or R_4 is apolar, one of the side-chains of a branched chain or one of the termini must be hydrophobic.

Chains in the substances of TWEEN type are very frequently of

the polyoxyethylene class. They mainly contain one terminal hydrogen atom and more rarely a methoxy group. One of the polyoxyethylene chains, however, contains a hydrophobic residue which preferably corresponds to an acyl-, alkyl-, alkenyl-, hydroxyalkyl-, hydroxyalkenyl- or hydroxyacyl-chain with 4-24, and in particular 12-18 carbon atoms.

Edge active substances which are sold under the trademark "TRITON" are also useful according to this invention.

Polyalcohol residues R_2 are most frequently esterified or etherified; however, in some cases they can also be bound to the hydrophobic chain through a nitrogen atom. They are very often adducts of ethyleneglycol, glycerol, erythritol, or pentaerythritol, for example 1-alkyl-, 1-alkenoyl-, 1-hydroxyalkene-glycerol, or corresponding 1,2-, or 1,3-diglycerides (for example, 1-alkyl,2-alkyl-, 1-alkyl,2-alkenyl-, 1-alkenyl,2-alkyl-, 1- alkenyl,2-alkenyl-, 1-alkenyl,2-hydroxyalkyl-, 1-hydroxyalkyl,2-alkenyl-, 1-alkyl,2-hydroxyalkyl-, 1-hydroxyalkyl,2-alkyl-, 1-alkenyl,2-hydroxyalkene-, 1-hydroxyalkene,3-alkenyl-, 1-alkyl,3-alkyl-, 1-alkyl,3-alkenyl-, 1-alkenyl,3-alkyl-, 1-alkenyl,3-alkenyl-, 1-alkenyl,3-hydroxyalkyl-, 1-hydroxyalkyl,3-alkenyl-, 1-alkyl,3-hydroxyalkyl-, 1-hydroxyalkyl,3-alkyl-, 1-alkenyl,3-hydroxyalkene- or 1-hydroxyalkene,3-alkenyl-). Glycerol can be replaced by another oligo- or polyalcohol, such as erythritol, pentantriol, hexantriol, -tetraol or -pentaol, etc., resulting in a wide variety of linkage possibilities.

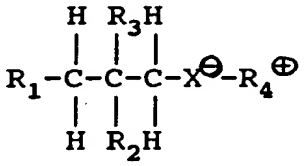
Z or R_2, moreover, can contain one or more 1-10, preferably 1-6, most frequently 1-3 carbohydrate residues or their derivatives. 'Carbohydrate residue' in this context has the meaning as already described and is an alpha or beta and L- or D-alloside, -altroside, -fucoside, -furanoside, -galactoside,

-galactopyranoside, -glucoside, -glucopyranoside, -lactopyranoside, -mannoside, -mannopyranoside, -psicoside, sorboside, -tagatoside, -taloside; frequently used derivatives of disaccharides are L- or D-maltopyranoside, -maltoside, -lactoside, malto- or -lactobionamide; the corresponding derivatives of maltotriose or -tetraose are also useful.

The carbohydrate residue can also contain a sulfur atom, e.g. in beta-L- or D-thioglucopyranoside or -thioglycoside.

Zwitterionic surfactants are substances, for example, which contain a sulphonate group, such as (3-((3-cholamidopropyl)-dimethylyammonio)-1-propanesulfonate (CHAPS) and (3-((3-cholamidopropyl)-dimethylyammonio)-2-hydroxy-1-propane-sulfonate (CHAPSO) or N-octyl-N,N-dimethyl-3-ammonio-1-propane-sulfonate, N-dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate (lauryl-sulfobetaine), N-tetradecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate (myristyl-sulfobetaine), N-hexadecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate (palmityl-sulfobetaine), N-octadecyl-N,N-dimethyl-3-ammonio-1-propane-sulfonate (stearyl-sulfobetaine), 'N-octadecenoyl-N,N,-dimethyl-3-ammonio-1-propanesulfonate (oleoyl-sulfobetaine) etc.

Zwitterionic surfactants are also substances with the basic formula 4



(4)

in which n is one or zero. One of both side chains R_1 and R_2 contains one acyl-, alkyl-, alkenyl-, alkenoyl-, hydroxyalkyl-, hydroxyalkenyl- or hydroxyacyl-, or alkoxy chain with 8-24 carbon atoms each; the other residue corresponds to a hydrogen, to a hydroxy group or to a short chain alkyl

residue. R_3 normally represents a hydrogen atom or a short alkyl chain. X is most frequently anionic, e.g. in a phosphate- or sulfate-residue. The residue R_4 in this case is cationic, in order to ensure that the whole molecule is zwitterionic. Most frequently, ammonio-alkyl derivatives, such as ethanol-, propanol-, butanol-, pentanolamine, hexanolamine, heptanolamine or octanolamine, N-methyl-, N,N-dimethyl, or N,N,N-trimethyl-ammonio-alkyl, N-ethyl-, N,N-diethyl, or N,N,N-triethyl-amino-alkyl, unequal N-alkyles, such as N,N-methyl-ethyl-ammonio-alkyl, or corresponding hydroxyalkyl substances are used, sometimes in a substituted form. (Single chain (lyso) derivatives of all biological zwitterionic phospholipids as well as their modified forms (such as Platelet-Activating-Factor and its analogs) also belong to this category.) R_4 can also be a positively charged carbohydrate residue, such as an aminosugar or one of its derivatives. R_4 and X, moreover, can exchange positions.

An ionic edge active substance is any material which contains at least one positive or negative charge and at least one segment which is poorly water soluble. An anionic substance of this kind can also contain several charges but must have a negative total charge. The total charge of any cationic substance must be positive.

Anionic edge active substances are for example the substances described by the basic formula 5:

$$\left[R_1 - \overset{\overset{O}{\|}}{C} - O \right]^{\ominus} G^{\oplus} \qquad (5)$$

in which R1 is an organic hydrocarbon residue, which can also be substituted, and G^+ is a monovalent counterion, chiefly an alkali metal cation (such as lithium, sodium, potassium,

rubidium, or cesium), an ammonium ion or a low weight tetraalkylammonium-ion, such as tetramethylammonium or tetraethylammonium.

The hydrocarbon residue R_1 in an anionic surfactant of the basic formula 5 is frequently a straight chain or branched acyl, alkyl or alkenoyl, or oxidized or hydroxygenated derivative thereof; the residue R_1 can also contain one or several cyclic segments.

R_1 chain frequently contains 6-24, more frequently 10-20, and most frequently 12-18 carbon atoms; if unsaturated, it contains 1-6, and even more frequently 1-3, double bonds in n-3- or n-6- position.

The following hydroxyalkyl chains are preferred for the present purpose: n-dodecylhydroxy (hydroxylauryl), n-tetradecylhydroxy (hydroxymyristyl), n-hexadecylhydroxy (hydroxycetyl), n-octadecylhydroxy (hydroxystearyl), n-eicosylhydroxy or n-docosyloxy. Amongst the hydroxyacyl chains, the hydroxylauroyl, hydroxymyristoyl, hydroxypalmitoyl, hydroxystearoyl, eicosoylhydroxy or docosoyloxy chains are especially worth mentioning; particularly interesting amongst the hydroxyalkene-residues are the hydroxydodecen, hydroxytetradecen, hydroxyhexadecen, hydroxyoctadecen, hydroxyeicosen, hydroxydocosen, most notably 9-cis,12-hydroxy-octadecenyl (ricinolenyl) or 9-trans,12-hydroxy-octadecenyl (ricinelaidyl), 5-cis,8-cis,11-cis,14-cis,15-hydroxy-eicosatetraenyl (15-hydroxy-arachidonyl), 5-cis,8-cis,11-cis,14-cis,15-hydroxy,17-cis-eicosapentaenyl, 4-cis,7-cis,10-cis,13-cis,15-hydroxy,16-cis-docosapentaenyl and 4-cis,7-cis,10-cis,13-cis,15-hydroxy,16-cis,19-cis-docosahexaenyl.

Another class of anionic, edge active substances corresponds

to basic formula 6

$$(R_1 - (O - X) - Y)^- G^+ \qquad (6)$$

here, R_1 is a hydrocarbon residue which can also be substituted; X is a short-chain alkyl residue and Y denotes a sulfonate-, sulfate-, phosphate-, phosphonate or phosphinate group. G^+ is a mostly monovalent counterion (cation).

Alkali metal alkyl- or -alkenylethersulfonates or -phosphates belong to this class of ether-bonded molecules. Special examples are sodium-or potassium-n-dodecyloxyethylsulfate, -n-tetradecyloxyethylsulfate, -n-hexadecyl-oxyethylsulfate or -n-octadecyloxyethylsulfate or an alkali metal alkane sulfonate, such as sodium- or potassium-n-hexanesulfonate, n-octansulfonate, n-decansulfonate, n-dodecansulfonate, -n-tetradecansulfonate, -n-hexadecansulfonate or -n-octadecan-sulfonate.

The substances of general formula 7

$$(R_1 - Y)^{\ominus} G^{\oplus} \qquad (7)$$

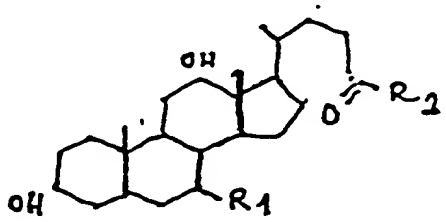
are related to the compounds of basic type 6. These are analogous to the substances of formula 6 but contain a directly (covalently) coupled charged headgroup.

Particularly useful anionic, edge active substances of above formula 6 are alkali metal-alkylsulfates. To mention just a few examples: sodium or potassium-n-dodecyl (lauryl)-sulfate, -n-tetradecyl (myristyl)-sulfate, -n-hexadecyl (palmityl)-sulfate, -n-octadecyl (stearyl)-sulfate, n-hexadecylen (palmitolein)-sulfate and n-octadecylen (olein)-sulfate. Instead of a sulfate group, sulfonate, n-methyl- or n-ethylglycine for example can also be used.

Various salts of bis-(2-alkyl-alkyl)-sulfosuccinate are also suitable for the applications as described in this work. Preferably, these are used as lithium-, sodium-, potassium-, or tetramethylammonium-bis-(2-ethyl-hexyl)-sulfosuccinate.

Furthermore, sarcosides, as well as alkyl- or alkenoyl-sulfochloride derivatives of the protein condensates, sulfonamide soaps, sulfatated or phosphorylated alcohol-esters, sulfatated or phosphorylated amides or monoglycerides, moreover, fatty acid alkylamides, sulfo- or phospho-succinic acid esters, taurides, alkylphenol-, alkylbenzol-, alkylnapthaline-ethersulfonates etc., are also all useful.

Another important group of anionic edge active substances are the derivatives of cholic acid. Their basic formula reads



$X = H, OH$

here, R_1 corresponds to a proton, an OH- or a carbonyl group and R_2 can be a derivative of taurine or glycocoll, for example. Particularly suitable are various salts of cholic acid (bile acid, 3alpha,7alpha,12alpha-trihydroxy-5beta-cholane-24-oin-acid), deoxycholic acid (3alpha,12alpha-dihydroxy-5beta-cholane-24-oin-acid), chenodeoxycholic acid, glycocholic acid (N-(3alpha,7alpha,12alpha-trihydroxy-24-oxycholane-24-yl-)glycine), deoxycholic acid, glycodeoxycholic acid (N-(3alpha,12alpha-dihydroxy-24-oxycholane-24-yl-) glycine), glycochenodeoxycholic acid, glycolitocholic acid, glycoursodeoxycholic acid, litocholic acid, taurodeoxycholic

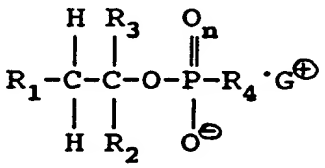
acid, taurocholic acid (3alpha,7alpha,12alpha-trihydroxy-5beta-cholan-24-oin-acid-N-(sulfoethyl)amide), taurochenodeoxycholic acid, tauroglycocholic acid, taurolitocholic acid, taurolitocholic acid-3-sulfate, tauroursodeoxycholic acid, ursocholanic acid, ursodeoxycholic acid (3alpha,7beta-dihydroxy-5beta-cholanic acid), the most common counterions being sodium or potassium.

Diverse cholic acid esters, such as cholesteryl-alkyl-, -alkenyl-, -hydroxyalkyl-, -hydroxyalkene-esters or cholesterylsulfates and -sulfonates are also edge active according to this invention.

Related synthetic adducts of the CHAPS class can also be used; in this case, R_2 is frequently an $NH-(CH_2)_3-N',N'-(CH_2)_2(CH_2)_2-R_3-CH_2-SO_3$ segment, whilst R_3 can be a proton or a carbonyl group. Again, sodium or potassium are the most commonly used counterions.

Digitonines as well as saponines, such as Quillaja acid, have similar basic structures in their cores as the cholic acid derivatives; consequently, they can also be used as edge active substances according to this invention.

The basic formula of the phosphorus-containing anionic edge active substances is



(8)

in which n is zero or one. One of the two side chains R_1 and R_2 contains hydrogen, a hydroxy group or a short chain alkyl residue; the other contains an alkyl-, alkenyl-, hydroxy-

alkyl-, hydroxyalkenyl- or hydroxyacyl-chain (or an alkenyl-, alkoxy-, alkenyloxy- or acyloxy-residue) with 8-24 carbon atoms. The R_3 residue, as a rule, corresponds to hydrogen or an alkyl chain with less than 5 carbon atoms. R_4 can be an anionic oxygen or a hydroxy group; an alkyl chain with up to 8 C-atoms can also appear as well as another carbohydrate residue with up to 12 carbon atoms; if R_1 as well as R_2 are hydrogen and/or hydroxy groups, a steroid residue, a sugar derivative, a chain containing an amino group, etc., can also appear. Alkyl residues can also be substituted.

Amongst the most suitable surfactants of this substance class are: n-tetradecyl(=myristoyl)-glycero-phosphatidic acid, n-hexadecyl-(=plamityl)-glycero-phosphatidic acid, n-octadecyl(=stearyl)-glycero-phosphatidic acid, n-hexadecylene(=palmitoleil)-glycero-phosphatidic acid, n-octadecylene(=oleil)-glycero-phosphatidic acid, n-tetradecyl-glycero,phosphoglycerol, n-hexadecyl-glycero-phosphoglycerol, n-octadecylene-glycero- phosphoglycerol, n-tetradecyl-glycero-phosphoserine, n-hexadecyl-glycerophosphoserine, -n-octadecyl-glycero-phosphoserine, n-hexadecylene-glycero-phosphoserine and n-octadecylene-glycero-phosphoserine.

The corresponding lyso-sulfolipids, phosphono- or phosphino-lipids are also suitable edge active compounds according to this invention.

Counterion in these compounds is most frequently an alkali metal cation (such as lithium, sodium, potassium, cesium) or a water soluble tetraalkylammonium-ion (such as tetramethyl-ammonium, tetrathylammonium, etc.).

All corresponding statements made above for surfactants of basic formula 3 also pertain to the carbohydrate residue R_1.

This residue in the majority of cases is a straight chain or branched alkyl or alkenoyl chain with 6-24, very frequently 10-20, in particular 12-18, carbon atoms and 1-6, especially frequently 1-3, double bonds in n-3- or n-6- positions.

Very convenient alkyl-residues R_1 or R_2 are, for example, n-dodecyl, n-tetradecyl, n-hexadecyl, n-octadecyl, n-eicosyl or n-docosyl chains. N-nonyl, n- undecyl, n-tridecyl, n-pentadecyl, n-heptadecyl and n-nonadecyl, however, are equally useful.

An alkenyl in position R_1 or R_2 is preferably a 9-cis-dodecenyl (lauroleyl), 9-cis-tetradecenyl (myristoleyl), 9-cis-hexadecenyl (palmitoleoyl), 6-cis-octadecenyl (petroselinyl), 6-trans-octadecenyl (petroselaidinyl), 9-cis-octadecenyl (oleyl), 9-trans-octadecenyl (elaidinyl), 11-cis-octadecenyl (vaccenyl), 9-cis-eicosenyl (gadoleinyl), 13-cis-docosenyl, 13-trans-docosenyl or 15-cis-tetracosenyl, etc.

Higher unsaturated alkenyls which also can be used for the present purpose are, amongst others: 9-cis,12-cis-octadecendienyl, 9-trans,12-trans-octadecendienyl, 9-cis,12-cis,15-cis-octadecentrienyl, 6-cis,9-cis,12-cis-octadecentrienyl, 11-cis,14-cis,17-cis-eicosatrienyl, 6-cis,9-cis,12-cis,15-cis-octadecentetraenyl, 5-cis,8-cis,11-cis,14-cis-eicosatetraenyl, 5-cis,8-cis,11-cis,14-cis,17-cis-eicosapentaenyl, 4-cis,7-cis,10-cis,13-cis,16-cis-docosapentaenyl and 4-cis,7-cis,10-cis,13-cis,16-cis,19-cis-docosahexaenyl.

R_1 and R_2 are preferably chosen from the substances of the hydroxyalkyl-class, in which case they correspond, for example, to n-decylhydroxy, n-dodecylhydroxy (hydroxylauryl), n-tetradecylhydroxy (hydroxymyristyl), n-hexadecylhydroxy (hydroxycetyl), n-octadecylhydroxy (hydroxystearyl) and n-

eicosylhydroxy (hydroxyarachinyl) chains.

An alkenylhydroxy in R_1 or R_2 is preferably a 9-cis-dodecenylhydroxy (hydroxylauroleyl), 9-cis-tetradecenylhydroxy (hydroxymyristoleyl), 9-cis-hexadecenylhydroxy (hydroxypalmitoleinyl), 6-cis-octadecenylhydroxy (petroselinylhydroxy), 6-trans-octadecenylhydroxy (hydroxypetroselaidinyl), 9-cis-octadecenylhydroxy (hydroxyoleyl), 9-trans-octadecenylhydroxy (hydroxyelaidinyl) and 9-cis-eicosenyl (hydroxygadoleinyl) chain.

An alkanoylhydroxy in R_1 or R_2 is preferably an n-decanoylhydroxy, n-dodecanoylhydroxy (lauroylhydroxy), n-tetradecanoylhydroxy (myristoylhydroxy), n-hexadecanoylhydroxy, n-hexadecanoylhydroxy (palmitoylhydroxy), n-octadecanoylhydroxy (stearoylhydroxy) and n-eicosoylhydroxy (arachinoylhydroxy) chain.

An alkenoylhydroxy in R_1 or R_2 is preferably a 9-cis-dodecenylhydroxy (lauroleoylhydroxy), 9-cis-tetradecenoylhydroxy (myristoleoylhydroxy), 9-cis-hexadecenoylhydroxy (palmitoleinoylhydroxy), 6-cis-octadecenoylhydroxy (peteroselinoylhydroxy), 6-trans-octadecenoylhydroxy (petroselaidinoylhydroxy), 9-cis-octadecenoylhydroxy (oleoylhydroxy), 9-trans-octadecenoylhydroxy (elaidinoylhydroxy) and 9-cis-eicosenoyl (gadoleinoylhydroxy) chain.

Some examples for the short chain alkyl residue, which often appear in the R_4 residue, are methylene-, ethylene-, n-propylene-, iso-propylene-, n-butylene- or iso-butylene- as well as n-pentylene- or n-hexylene-groups. R_4 can also be a carboxy- or a sulfo-group, an acid or alkaline group, such as carboxy- and amino-group; the amino group in such case is always in the alpha-position relative to the carboxy group.

Another example for the R_4 residue are free or etherified hydroxy groups (two ether-bonded hydroxy groups, in such case, can be connected by one divalent hydrocarbon residue, such as methylene, ethylene, ethylidene, 1,2-propylene or 2,2-propylene). R_4, furthermore, can be substituted by a halogen atom, such as chlorine or bromine, a low weight alkoxy-carbonyl, such as methoxy- or ethoxycarbonyl, or by a low weight alkansulfonyl-, such as methansulfonyl.

A substituted short chain alkyl residue R_4 with 1-7 C-atoms is preferably carboxy-short-chain alkyl, such as carboxy-methyl, carboxyethyl- or 3-carboxy-n-propyl, omega-amino-n-carboxy- a short-chain alkyl, such as 2-amino-2-carboxyethyl or 3-amino-3-carboxy-n-propyl, hydroxy-short-chain alkyl, such as 2-hydroxyethyl or 2,3-dihydroxypropyl, a short-chain alkoxy-3-methoxy-n-propyl, a short-chain alkylendioxy-short-chain alkyl, such as 2,3-ethylenedioxypropyl or 2,3-(2,2-propylene)-dioxypropyl, or halogen-short-chain alkyl, such as chloro- or bromo-methyl, 2-chloro- or 2-bromo-ethyl, 2- or 3-chloro- or 2-or 3-bromo-n-propyl.

A carbohydrate residue R_4 with 5-12 C-atoms is, for example, a natural monosaccharide residue stemming from a pentose or a hexose in the aldose or ketose form.

A carbohydrate residue R_4, moreover, can be a natural disaccharide residue, such as a disaccharide residue formed from two hexoses, in the described sense. A carbohydrate residue R_4 can also be a derivatised mono-, di- or oligosaccharide residue, in which an aldehyde group and/or one or two terminal hydroxy groups are oxidized to a carboxy group, e.g. a D-glucon-, D-glucar- or D-glucoron acid residue; this preferably appears in the form of a cyclic lactone residue. The aldehyde- or keto-groups in a derivatised mono-

or disaccharide residue can also be reduced to a hydroxy group, e.g. in inositol, sorbitol or D-mannitol; also, one or several hydroxy groups can be replaced by a hydrogen atom, e.g. in desoxysugars, such as 2-desoxy-D-ribose, L-rhamnose or L-fucose, or by an amino group, e.g. in aminosugars, such as D-glucosamine or D-galactosamine.

R_4 can also be a steroid residue or a sterine residue. If R_4 is a steroid residue, R_3 is a hydrogen atom, whilst R_1 and R_2 in such case preferably correspond to a hydroxy group.

The counterion in such cases is preferably an ammonium, sodium or potassium ion.

In an anionic surfactant of formula 8, the following values of parameters are preferred: n = 1, R_1 is an alkyl, such as n-dodecyl (lauryl), n-tridecyl, n-tetradecyl (myristyl), n-pentadecyl, n-hexadecyl (cetyl), n-heptadecyl or n-octadecyl (stearyl), hydroxyalkyl, such as n-dodecylhydroxy (hydroxy-lauryl), n-tetradecylhydroxy (hydroxymyristyl), n-hexadecyl-hydroxy (hydroxycetyl), or n- octadecylhydroxy (hydroxy-stearyl), hydroxyacyl, such as hydroxylauroyl, hydroxy-myristoyl, hydroxypalmitoyl or hydroxystearoyl, R_2 is a hydrogen atom or a hydroxy group, R_3 is a hydrogen atom or a short-chain alkyl, such as methyl, R_4 is a short-chain alkyl, e.g. methyl or ethyl, short-chain alkyl substituted by an acid or an alkaline group, such as a carboxy and amino group, e.g. omega-amino-omega-carboxy-short-chain alkyl, such as 2-amino-2-carboxyethyl or 3-amino-3-carboxy-n-propyl, hydroxy-short-chain alkyl, such as 2-hydroxyethyl or 2,3-hydroxypropyl, short-chain alkylenedioxy-short-chain alkyl, e.g. 2,3-ethylenedioxypropyl or 2,3-(2,2-propylene)-dioxypropyl, halogen-short-chain alkyl, such as 2-chloro- or 2-bromo-ethyl group, a carbohydrate residue with 5-12 C-

atoms, e.g. in inositol, or a steroid residue, such as a sterol, e.g. cholesterin, and G^+ is a sodium-, potassium- or ammonium-ion.

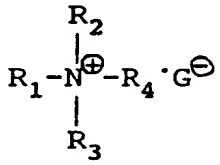
An anionic surfactant of formula 8, in many cases, is a sodium- or potassium salt of lysophosphatidylserine, such as the sodium- or potassium salt of lysophosphatidylserine from bovine brain or the sodium- or potassium salt of a synthetic lysophosphatidylserine, such as sodium- or potassium-1-myristoyl- or -1-palmitoyl-lysophosphatidylserine, or a sodium- or potassium salt of lysophosphatidylglycerols. The hydrogen atom on the phosphate group can be replaced by a second cation, G^+ or calcium-, magnesium-, manganese-ion, etc.

An anionic surfactant of formula 8 preferably contains an alkyl chain, such as n-dodecyl (lauryl), n-tridecyl, n-tetradecyl (myristoyl), n-pentacedyl, n-hexadecyl (cetyl), n-heptadecyl or n-octadecyl (stearyl), a hydroxyalkyl chain, such as n-dodecylhydroxy (hydroxylauryl), n-tetradecylhydroxy (hydroxymyristyl), n-hexadecylhydroxy (hydroxycetyl), or n-octadecylhydroxy (hydroxystearyl), a hydroxyacyl chain, such as hydroxylauroyl, hydroxymyristoyl, hydroxypalmitoyl or hydroxystearoyl in position R_1, a hydrogen atom or a hydroxy group in position R_2, and a hydrogen atom or a short-chain alkyl, such as methyl group, in position R_3. G^+ is preferably an ammonium, sodium, potassium or tetramethylammonium ion.

An anionic surfactant of formula 8 can, furthermore, be a sodium- or potassium salt of a natural phosphatidic acid, such as egg-phosphatidic acid, a sodium- or potassium salt of a natural lysophosphatidic acid, such as egg-lysophosphatidic acid, a sodium- or potassium salt of a synthetic lysophosphatidic acid, such as 1-lauroyl-, 1-myristoyl-, 1-palmitoyl- or 1-oleoyl-lysophosphatidic acid, etc.

The most important classes of cationic surfactants encompass: ammonium salts, quarternary ammonium salts, salts of heterocyclic bases, such as alkylpyridium-, imidazole-, or imidazolinium salts, salts of alkylamides and polyamines, salts of acylated diamines and polyamines, salts of acylated alkanolamines, salts of alkanolamine esters and ethers, etc.

A cationic surfactant is, for example, any substance corresponding to the formula:



(9)

in which R_1 is a hydrocarbon residue which can also be substituted. R_2 denotes a short-chain alkyl, phenyl-short-chain-alkyl or hydrogen atom. R_3 and R_4 correspond to a short-chain alkyl residue. R_2 and R_3, together with the nitrogen atom, represent an aliphatic heterocycle, which can also be substituted on a carbon atom; R_4 is a short-chain alkyl; R_2, R_3 and R_4, together with the nitrogen atom, can also form an aromatic heterocycle, which, moreover, can be substituted on one of the carbon atoms. G^- corresponds to an anion.

In a cationic surfactant of basic formula 9, R_1 represents an aliphatic hydrocarbon residue, which can also be substituted, for example, by an aryloxy- short-chain-alkoxy-, a substituted short-chain alkyl, a straight chain or branched chain alkyl with 7-22, and in particular 12-20, carbon atoms, or an alkenyl with 8-20, or in particular 12-20, carbon atoms and 1-4 double bonds.

Particularly preferred for use are straight chain alkyles with an even number of 12-22 carbon atoms, such as n-dodecyl, n-tetradecyl, n-hexadecyl, n-octadecyl, n-eicosyl or n-docosyl.

An alkenyl with 8-24, in particular 12-22, carbon atoms and 0-5, in particular 1-3, double bonds is e.g. 1-octenyl, 1-nonenyl, 1-decenyl, 1-undecenyl, 1-dodecenyl, 9- cis-dodecenyl (lauroleyl), 1-tridecenyl, 1-tetradecenyl, 9-cis-tetradecenyl (myristoleyl), 1-pentadecenyl, 1-hexadecenyl, 9-cis-hexadecenyl (palmitoleinyl), 1-heptadecenyl, 1-octadecenyl, 6-cis-octadecenyl (petroselinyl), 6-trans-octadecenyl (petroselaidinyl), 9-cis-octadecenyl (oleyl), 9-trans-octadecenyl (elaidinyl), 9-cis-12-cis-octadecadienyl (linoleyl), 9-cis-11-trans-13-trans-octadecatrienyl (alpha-elaostearinyl), 9-trans-11-trans-13-trans-octadecatrienyl (beta-elaostearinyl), 9-cis-12-15-cis-octadecatrienyl (linolenyl), 9-, 11-, 13-, 15-octadecatetraenyl (parinaryl), 1-nonadecenyl, 1-eicosenyl, 9-cis-eicosenyl (gadoleinyl), 5-, 11-, 14-eicosatrienyl or 5-, 8-, 11-, 14-eicosatetraenyl (arachidonyl).

Preferred alkenyls contain 12-20 carbon atoms and one double bond, e.g. 9-cis-dodecenyl (lauroleyl), 9-cis-tetradecenyl (myristoleyl), 9-cis-hexadecenyl (palmitoleinyl), 6-cis-octadecenyl (petroselinyl), 6-trans-octadecenyl (petroselaidinyl), 9-cis-octadecenyl (oleyl), 9-trans-octadecenyl (elaidinyl) or 9-cis-eicosenyl (gadoleinyl).

Methyl or ethyl are two examples of short-chain alkyl residues R_2, R_3 or R_4 which appear in substances of formula 9.

Two examples of phenyl-short-chain-alkyl groups in R_2 are benzyl or 2-phenylethyl.

An aliphatic heterocycle, which can form from R_2 and R_3 together with the nitrogen atom is, for example, a monocyclic, five- or six-member aza-, oxaaza- or thiazacyclyl residue, as in piperidino, morpholino or thiamorpholinio groups.

Substituents of this heterocycle are the substituents R_1 and R_4 on the nitrogen as well as, in some cases, on the carbon atom; they are, most frequently, of the short-chain alkyl, such as methyl, ethyl, n-propyl or n-butyl type.

A heterocycle, which is formed from R_2 and R_3 together with nitrogen and is substituted on a carbon atom through a short-chain alkyl, is e.g. of the 2-, 3- or 4-methylpiperidinio, 2-, 3- or 4-ethylpiperidinio or 2- or 3-methylmorpholinio type.

An aromatic heterocycle, formed from R_2, R_3 and R_4 together with the nitrogen atom, is, for example, a monocyclic five- or six-member aza-, diaza-, oxaaza- or thiazacyclyl residue, such as pyridinio, imidazolinio, oxazolinio or thiazolinio or, for example, a benzocondensed monoazabicyclyl residue, such as chinolinio or iso-chinolinio group.

Substituents of such heterocycles are the residue R_1 on the nitrogen atom as well as a short-chain alkyl, such as methyl or ethyl, hydroxy-short-chain alkyl, such as hydroxymethyl or 2-hydroxyethyl, oxo-, hydroxy- or halogen, such as chloro- or bromo-compounds, which can also be substituted on a carbon atom.

A heterocycle, formed from R_2, R_3 and R_4 and substituted on a carbon atom through the mentioned residues is, for example, a 2- or 4-short-chain-alkylpyridinio, e.g. 2- or 4-methyl or 2- or 4-ethylpyridinio, di-short-chain-alkylpyridinio, e.g. 2,6-

dimethyl-, 2-methyl-3-ethyl-, 2-methyl-4-ethyl-, 2-methyl-5-ethyl-, or 2-methyl-6-ethylpyridinio, 2-, 3-or 4-halogen-pyridinio, e.g. 2-, 3- or 4-chloropyridinio or 2-, 3- or 4-bromo-pyridinio, 2-short-chain alkylimidazolinio, -oxazolinio or -thiazolinio, such as 2-methyl- or 2-ethylimidazolinio, -oxazolinio or -thiazolinio or 2-short-chain alkyl-8-halogenchinolinio, such as 2-methyl-8-chlorochinolinio group.

A cationic surfactant of formula 9 is preferably an N-benzyl-N,N-dimethyl-N-2-(2-(4-(1,1,3,3-tetramethylbutyl)-phenhydroxy)-ethhydroxy)-ethylammoniochloride, N-benzyl-N,N-dimethyl-N-2-(2-(3(methyl-4-(1,1,3,3-tetramethylbutyl)-phenhydroxy)-ethhydroxy)-ethylammoniochloride (methylbenzethoniumchloride), n-dodecyltrimethylammonio-chloride or -bromide, trimethyl-n-tetradecylammoniochloride or -bromide, n-hexadecyltrimethylammoniochloride or -bromide (cetyltrimethyl-ammoniumchloride or -bromide), trimethyl-n-octadecylammoniochloride or -bromide, ethyl-n-dodecyl-dimethylammoniochloride or -bromide, ethyldimethyl-n-tetradecylammoniochloride or -bromide, ethyl-n-hexadecyldimethylammoniochloride or -bromide, ethyldimethyl-n-octadecylammoniochloride or -bromide, n-alkyl-benzyl-dimethyl-ammoniochloride or -bromid (benzalkoniumchloride or -bromide), such as benzyl-n-dodecyldimethylammoniochloride or bromide, benzyldimethyl-n-tetradecylammoniochloride or -bromide, benzyl-n-hexadecyldimethyl-ammoniochloride or -bromide or benzyldimethyl-n-octadecylammonio-chloride or -bromide, N-(n-decyl)-pyridiniochloride or -bromide, N-(n-dodecyl)-pyridiniochloride or -bromide, N-(n-tetradeyl)-pyridiniochloride or -bromide, N-(n-hexadecyl)-pyridiniochloride or -bromide (cetylpyridiniumchloride) or N-(n-octadecyl)-pyridinio-chloride or -bromide. Mixtures of these or other edge active substances are also suitable.

The following surfactants are especially useful for biological

purposes: N,N-bis(3-D-glucon-amidopropyl)cholamide (BigCHAP), Bis(2-ethylhexyl)sodium-sulfosuccinate, cetyl-trimethyl-ammonium-bromide, 3-((cholamidopropyl)-dimethylammonio)-2-hydroxy-1-propane sulfonate (CHAPSO), 3-((cholamidopropyl)-dimethylammonio)-1-propane sulfonate (CHAPS), cholate-sodium salt, decaoxyethylene-dodecyl-ether (Genapol C-100), decaethylene-isotridecyl-ether (Genapol X-100), decanoyl-N-methyl-glucamide (MEGA-10), decyl-glucoside, decyl-maltoside, 3-(decyldimethylammonio)-propane-sulfonate (Zwittergent 3-10), deoxy-bigCHAP, deoxycholate, sodium salt, digitonin, 3-(dodecyldimethylammonio)-propane-sulfonate (Zwittergent 3-12), dodecyl-dimethyl-amine-oxide (EMPIGEN), dodecyl-maltoside, dodecylsulfate, glyco-cholate, sodium salt, glyco-deoxycholate, sodium salt, heptaethylene-glycol-octyl-phenyl-ether (triton X-114), heptyl-glucoside, heptyl-thioglucoside, 3-(hexadecyldimethylammonio)-propane-sulfonate (Zwittergent 3-14), hexyl-glucoside, dodecyl-dimethyl-amine-oxide (Genaminox KC), N-dodecyl-N,N-dimethylglycine (Empigen BB), N-decyl-sulfobetaine (Zwittergent 3-10), N-dodecyl-sulfobetaine (Zwittergent 3-12), N-hexadecyl-sulfobetaine (Zwittergent 3-16), N-tetradecyl-sulfobetaine (Zwittergent 3-14), N-octyl-sulfobetaine (Zwittergent 3-08), nonaethylene-glycol-mono-dodecyl-ether (THESIT), nonaethylene-glycol-octyl-phenol-ether (triton X-100), nonaethylene-glycol-octyl-phenyl-ether (NP-40, Nonidet P-40), nonaethylene-dodecyl-ether, nonanoyl-N-methyl-glucamide (MEGA-9), nonaoxyethylene-dodecyl-ether (Lubrol PX, Thesit), nonyl-glucoside, octaethylene-glycol-isotridecyl-ether (Genapol X-080), octaethylene-dodecyl-ether, octanoyl-N-methyl-glucamide (MEGA-8), 3-(octyldimethylammonio)-propane-sulfonate (Zwittergent 3-08), octyl-glucoside, octyl-thioglucoside, entadecaethylene-isotridecyl-ether (Genapol X-150), polyethylene-polypropylene-glycol (Pluronic F-127), polyoxyethylene-sorbitane-monolaurate (Tween 20), polyoxy-ethylene-sorbitane-monooleate (Tween 80), taurodeoxycholate-sodium salt, taurocholate-sodium salt, 3-(tetradecyl-

dimethylammonio)-propane-sulfonate (Zwittergent 3-14), etc.

Particularly suitable for pharmacological purposes are: cetyl-trimethyl-ammonium-salts (such as hexadecyltrimethylammoniumbromide, trimethylhexadecylamine-bromo-salt), cetylsulfate salts (such as Na-salt, Lanette E), cholate salts (such as Na- and ammonium-form) decaoxyethylene-dodecyl-ether (Genapol C-100), deoxycholate salts, dodecyl-dimethyl-amine-oxide (Genaminox KC, EMPIGEN), N-dodecyl-N,N-dimethylglycine (Empigen BB), 3-(hexadecyldimethylammonio)-propane-sulfonate (Zwittergent 3-14), fatty acid salts and fatty alcohols, glyco-deoxycholate salts, laurylsulfate salts (sodium dodecylsulfate, Duponol C, SDS, Texapon K12), N-hexadecyl-sulfobetaine (Zwittergent 3-16), nonaethylene-glycol-octyl-phenyl-ether (NP-40, Nonidet P-40), nonaethylene-dodecyl-ether, octaethylene-glycol-isotridecyl-ether (Genapol X-080), octaethylene-dodecyl-ether, polyethylene glycol-20-sorbitane-monolaurate (Tween 20), polyethylene glycol-20-sorbitane-monostearate (Tween 60), polyethylene glycol-20-sorbitane-monooleate (Tween 80), polyhydroxyethylene-cetylstearylether (Cetomacrogo, Cremophor O, Eumulgin, C 1000) polyhydroxyethylene-4-laurylether (Brij 30), polyhydroxy-ethylene-23-laurylether (Brij 35), polyhydroxyethylene-8-stearate (Myrj 45, Cremophor AP), polyhydroxyethylene-40-stearate (Myrj 52), polyhydroxyethylene-100-stearate (Myrj 59), polyethoxylated castor oil 40 (Cremophor EL), polyethoxylated hydrogenated castor oil (Cremophor RH 40, Cremophor RH 60) polyethoxylated plant oils (Lebrafils), sorbitane-monolaurate (Arlacel 20, Span 20), taurodeoxycholate salts, taurocholate salts, polyethylene glycol-20-sorbitane-palmitate (Tween 40), Myrj 49 and polyethylene glycol derivatives of ricinols, etc.

AGENTS:

Transfersomes as described in this invention are suitable for the application of many different agents and, in particular, for therapeutic purposes, for example. The preparations according to this invention can contain the following:

- at least one adrenocorticostatic agent, in particular metyrapon;

- at least one carrier substance, additive or agent, belonging to the class of beta-adrenolytics (beta blocking agents), very frequently acetobol, alprenolol, bisoprololfumarate, bupranolol, carazolol, celiprolol, mepindolsulfate, metipranolol, metoprolotartat, nadolol, oxyprenolol, pindolol, sotalol, tertatolol, timolohydrogen maleate and toliprolol, especially preferred, atenolol or propranolol;

- at least one carrier substance, additive or agent, belonging to the androgenes or antiandrogenes, in particular drostanolonpropionate, mesterolon, testosteronundecanoate, testolacton, yohimbine, or chloroamidinonacetate, cyproteronacetate, ethinylestradiol or flutamide;

- at least one carrier substance, additive or agent with an antiparasitic action, frequently phanquinone, benzyo-benzoate, bephenium-hydroxy-naphthoate, crotamitone, diethylcarbamazine, levamisol, lindane, malathione, mesulfene (2,7-dimethylantren), metronidazol or tetramisol;

- at least one anabolic agent, in particular clostebolacetate, cyanocobolamine, folic acid, mestanolone, metandienone, metenolone, nandrolone, nandrolondecanoate, nandrolone-hexyloxyphenylpropionate,

nandrolon-phenyl-propionate, norethandrolone, oxaboloncipionate, piridoxine or stanozolole;

- at least one agent which can induce systemic anesthesia or analgesia, e.g. chlorobutanol, ketamine, oxetacaine, propanidide and thiamylal, aminophenol-derivatives, aminophenazol-derivatives, antranilic acid- and arylpropione acid derivatives, azapropazone, bumadizone, chloroquin- and codeine-derivatives, diclophenac, fentanil, ibuprofen, indometacine, ketoprofen, methadone-substances, morazone, morphine and its derivatives, nifenazone, niflumin acid, pentazozine, pethidine, phenazopyridine, phenylbutazone-derivatives (such as 3,5 pyrazolidine dion), pherazone, piroxicam, propoxyphene, propyphenazon, pyrazol- and phenazone-derivatives (aminophenazone, metamizole, monophenylbutazone, oxyphenebutazone, phenylbutazone or phenazone-salyzilate), salicylic acid-derivatives, sulfasalazine, tilidine; acetylsalicylic acid, ethylmorphine, alclofenac, alphaprodine, aminophenazone, anileridine, azapropazone, benfotiamine, benorilate, benzydamine, cetobemidone, chlorophenesincarbamate, chlorothenoxazine, codeine, dextromoramide, dextro-propoxyphene, ethoheptazine, fentanyl, fenyramidol, fursultiamine, flupirtinmaleate, glafenine, hydromorphone, lactylphenetidine, levorphanol, mefenamic acid, meptazonol, methadone, mofebutazone, nalbufine, Na-salt of noramidopyrinium-methanesulfonate, nefopam, normethadone, oxycodone, paracetamol, pentazocine, pethidine, phenacetine, phenazocine, phenoperidine, pholcodine, piperylone, piritramide, procaine, propyphenazone, salicylamide, thebacone, tiemonium-odide, tramadone;

- at least one substance from the class of analeptics, such

as aminophenazole, bemegride, caffeine, doxapram, ephedrine, prolintane, or nialamide and tranylcypromine; but also vitamins, plant extracts from semen colae, camphor, menthol;

- at least one substance from the class of antiallergics: e.g. agents from the globuline family, corticoids or antihistaminics (such as beclometasone-, betametasone-cortisone-, dexametasone-derivatives, etc.) as well as bamipinacetate, buclizine, clemastine, clemizole, cromoglicinic acid, cyproheptadine, diflucorolonvalerate, dimetotiazine, diphenhydramine, diphenylpyraline, ephedrine, fluocinolane, histapyrrodine, isothipendyle, methadilazine, oxomemazine, paramethasone, prednilidene, theophilline, tolpropamine tritoqualine, etc. are used; amongst the preferred agents in this class are the substances characterized by their capacity to interfere (stimulate or suppress) the production of immunologically active substances, such as interleukines, interferones, leucotrienes, prostaglandines, etc. Amongst others, certain lipids and lipoids, such as phosphatidylcholines and diacylglycerols, or fatty acids and their esters, with chains containing several, preferably 3-6, most very frequently 3 or 4, double bonds, preferably of the n-3 type, are used for this purpose; the latter may also be hydroxygenated, branched or (partially) derivatized into ring structures.

- at least one substance with antiarrhythmic action, such as most of the cardiacs and beta-blockers, ajmaline, bupranolol, chinidine, digoxine derivatives, diltiazem, disopyramidedihydrogensulfate, erythromycine, disopyramide, gallopamil, ipratropiumbromide, lanatoside, lidocaine, lorcainide, orciprenalinesulfate, procaine amide, propafenone, sparteinesulfate, verapamil,

toliprolol.

- an antiarteriosclerotic, such as clofibrate.

- at least one substance belonging to the antiasthmatics and/or bronchospasmolytics, such as amiodarone, carbuterol, fenoterol, orciprenalin, sotalol, or theophilline-derivatives, as well as corticoids (such as beclomethasone, dexamethasone, hydrocortisone, prednisolone), frequently in combination with purines;

- at least one substance from the class of antibiotics, such as actinomycine, alamethicine, alexidine, 6-aminopenicillanic acid, moxicilline, amphotericine, ampicilline, anisomycine, antiamoebine, antimycine, aphidicoline, azidamfenicol, azidocilline, bacitracine, beclomethasone, benzathine, benzylpenicilline, bleomycine, bleomycine sulfate, calcium ionophor A23187, capreomycine, carbenicilline, cefacetril, cefaclor, cefamandole nafate, cefazoline, cefalexine, cefaloglycine, cefaloridine, cefalotine, cefapirine, cefazoline, cefoperazone, ceftriaxone, cefuroxim, cephalexine, cephaloglycine, cephalothine, cephapirine, cerulenine, chloroamphenicol, chlorotetracycline, chloroamphenicol diacetate, ciclaciline, clindamycine, chloromadinone acetate, chloropheniramine, chromomycine A3, cinnarizine, ciprofloxacine, clotrimazole, cloxacilline, colistine methanesulfonate, cycloserine, deacetylanisomycine, demeclocycline, 4,4'-diamino-diphenyl sulfone, diaveridine, dicloxacilline, dihydrostreptomycine, dipyridamol, doxorubicine, doxycycline, epicilline, erythromycine, erythromycine-stolate, erythromycinethylsuccinate, erythromycine stearate, ethambutol, flucloxacilline, fluocinolone acetonide, 5-fluorocytosine, filipine, formycine,

fumaramidomycine, furaltadone, fusidic acid, geneticine, gentamycine, gentamycine sulfate, gliotoxine, gfamicidine, griseofulvine, helvolic acid, hemolysine, hetacillin, kasugamycine, kanamycine (A), lasalocide, lincomycine, magnesidine, melphalane, metacycline, meticilline, mevinoline, micamycine, mithramycine, mithramycine A, mithramycine complex, mitomycine, minocycline, mycophenolic acid, myxothiazol, natamycine, nafcilline, neomycine, neomycine sulfate, 5-nitro-2-furaldehydesemicarbazone, novobiocine, nystatine, oleandomycine, oleandomycine phosphate, oxacihine, oxytetracycline, paromomycine, penicilline, pecilocine, pheneticilline, phenoxymethylpenicilline, phenyl amino-salicylate, phleomycine, pivampicilline, polymyxine B, propicilline, puromycine, puromycine aminonucleoside, puromycine aminonucleoside 5'-monophosphate, pyridinol carbamate, rolitetracycline, rifampicine, rifamycine B, rifamycine SV, spectinomycine, spiramycine, strepto-mycine, streptomycine sulfate, sulfabenzamide, sulfadimethoxine, sulfamethizol, sulfamethoxazol, tetracycline, thiamphenicol, tobramycine, troleando-mycine, tunicamycine, tunicamycine A1-homologs, tunicamycine A2-homolog, valinomycine, vancomycine, vineomycine A1, virginiamycine M1, viomycine, xylostasine;

- at least one substance with an antidepressive or antipsychotic action, such as diverse monoaminoxidase-suppressors, tri- and tetracyclic antidepressives, etc. Very frequently used agents of this class are alprazolame, amitriptyline, chloropromazine, clomipramine, desipramine, dibenzepine, dimetacrine, dosulepine, doxepine, fluvoxaminhydrogenmaleate, imipramine, isocarboxazide, lofepramine, maprotiline, melitracene, mianserine, nialamide, noxiptiline,

nomifensine, nortriptyline, opipramol, oxypertine, oxytriptane, phenelzine, protriptyline, sulpiride, tranylcypromine, trosadone, tryptophane, vitoxazine, etc.

- at least one antidiabetic agent, such as acetohexamide, buformine, carbutamide, chloropropamide, glibenclamide, glibornuride, glymidine, metformine, phenformine, tolazamide, tolbutamide;

- at least one substance acting as an antidote, for example, against the heavy metal poisoning, poisoning with insecticides, against drugs, blood poisons, etc. A few examples are different chelators, amiphenazol obidoxim-chloride, D-penicillamine, tiopromine, etc.;

- at least one substance from the class of antiemetics: some of such suitable agents are alizapride, benzquinamide, betahistidine-derivatives, cyclizine, difenidol, dimenhydrinate, haloperidol, meclozine, metoclopramide, metopimazine, oxypendyl, perphenazine, pipamazine, piprinhydrinate, prochloroperazine, promazine, scopolamine, sulpiride, thiethylperazine, thioproperazine, triflupromazine, trimethobenzamide, etc., which are frequently used in combination with vitamins and/or antiallergics;

- at least one substance with an antiepileptic action, such as barbexaclone, barbiturate, beclamide, carbamazepine, chloroalhydrate, clonazepam, diazepam, ethosuximide, ethylphenacemide, lorazepam, mephenytoine, mesuximide, oxazolidine, phenaglycodol, phensuximide, phenytoine, primidone, succinimide-derivatives, sultiam, trimethadione, yalproinic acid, etc.; additives are commonly chosen from the classes of hypnotics and sedatives; an especially commonly used agent of this kind is

carbamazepine.

- at least one substance with antifibrinolytic activity, such as aminocapronic acid or tranexamic acid.

- at least one anticonvulsive agent, such as beclamide, carbamazepine, clomethiazole, clonazepam, methylphenobarbital, phenobarbital or sultiam;

- at least one substance which modifies choline concentration, by having an anticholinergic activity, for example. The following substances can be used, amongst others, as cholinergics: aubenoniumchloride, carbachol, cerulezide, dexpanthenol and stigmine-derivatives (such as distigminebromide, neostigminemethylsulfate, pyridostigmine-bromide); frequently used as anticholinergics are especially atropine, atropinmethonitrate, benactyzine, benzilonium-bromide, bevonium-methylsulfate, chlorobenzoxamine, ciclonium-bromide, clidinium-bromide, dicycloverine, diphemanil-methylsulfate, fenpiverinium-bromide, glycopyrroniumbromide, isopropamide-iodide, mepenzolate-bromide, octatropine-methylbromide, oxyphencyclimine, oxyphenonium-bromide, pentapiperide, pipenzolate-bromide, piperidolate, pridinol, propanidide, tridihexethyl-iodide and trospiumchloride; cholinesterase inhibitors, such as ambenonium-chloride, demecarium-bromide, echothiopate-iodide, etc., are also useful for this purpose;

- at least one substance which can change, in the majority of cases diminish, the effect or concentration of histamine (antihistaminics). Preferred are hypoallergic carriers or hypoallergic edge active substances with n-3 (omega-3), less frequently with n-6 (omega-6), and mainly several, often 3-6 double bonds; such substances are

occasionally employed with hydroxy, more rarely methyl-, or oxo-side groups, or in an epoxy configuration; further suitable agents of this class are, among other substances, aethylenediamine, alimemazine, antazoline, bamipine, bromo-azine, bromo-pheniramine, buclizine, carbinoxamine, chlorocyclizine, chloropyramine, chlorophenanine, chlorophenoxamine, cimetidine, cinnarizine, clemastine, clemizol, colamine (such as diphenhydramine), cyclizine, dexbrompheniramine, dexchloropheniramine, difenidol, dimetindene, dimetotiazine, diphenhydramine, diphenylpyraline, dixyrazine, doxylamine, histapyrrodine, isothipendyl, mebhydroline, meclozine, medrylamine, mepyramine, methdilazine, pheniramine, piperacetazine, piprinhydrinate, pyrilamine (mepyramine), promethazine, propylamine, pyrrobutanine, thenalidine, tolpropamine, tripelennamine, triprolidine, etc.;

- at least one substance belonging to the class of antihypertonics, such as many alpha-receptor agonists, aldosterone-antagonists, angiotensine-converting-enzyme-blockers, antisymphaticotonics, beta-blockers, calcium-antagonists, diuretics, vasodilators, etc.; suitable agents for this purpose are for example alpenolol, atenolol, bendroflumethiazide, betanidine, butizide, chlorotalidone, clonidine, cycletanine, cyclopenthiazide, debrisoquine, diazoxide, dihydralazine, dihydroergotaminmethanesulfonate, doxazinmesilate, guanethidine, guanoclor, guanoxane, hexamethonium-chloride, hydralazine, labetalol, mecanylanine, methyldopa, pargyline, phenoxybenzamine, prazosine, quinethazone, spironolactone, bescinnamine, reserpine, trichloromethiazide or vincamine;

- at least one substance which is an inhibitor of biological activity, such as actinomycine C1, alpha-

amanitine, ampicilline, aphidicoline, aprotinine, calmidazolium (R24571), calpaine-inhibitor I, calpaine-inhibitor II, castanospermine, chloroamphenicol, colcemide, cordycepine, cystatine, 2,3-dehydro-2-desoxy-n-acetyl-neuraminic acid, 1-desoxymannojirimycine-hydrochloride, 1-desoxynojirimycine, diacylglycerol-kinase-inhibitor, P1, P5-di(adenosine-5'-)-penta-phosphate, ebelactone A, ebelactone B, erythromycine, ethidiumbromide, N-hydroxyurea, hygromycine B, kanamycine sulfate, alpha2-macroglobuline, N-methyl-1-desoxynojiri-mycine, mitomycine C, myxothiazol, novobiocine, phalloid-ine, phenylmethylsulfonylfluoride, puromycine-dihydro-chloride, rifampicine, staurosporine, streptomycine sulfate, streptozotocine, G-strophanthine, swainsonine, tetracycline-hydrochloride, trifluoperazine-dihydro-chloride, tunicamycine, etc.; useful proteinase inhibitors are, for example, (4-amidinophenyl)-methanesulfonylfluoride (APMSF), antipaine-dihydro-chloride, antithrombine III, alpha-1-antitrypsine, aprotinine, bestatine, calpaine-inhibitor I, calpaine-inhibitor II, L-1-chloro-3-(4-tosylamido)-7-amino-2-heptanone-hydrochloride (TLCK), L-1-chloro-3-(4-tosylamido)-4-phenyl-2-butanone (TPCK), chymostatine, cystatine, 3,4-dichlorisocoumarin, E 64, selastatinal, hirudin, kallikrein-inhibitor (aprotinine) L-leucinthiol, leupeptine, pepstatine, phenylmethylsulfonylfluoride (PMSF), phosphoramidone, TLCK (tosyl-lysine-chloromethyl-ketone), TPCK (tosyl-phenylalanine-chloromethyl-ketone), trypsine-inhibitors, etc.;

- at least one substance acting as an antihypotonic agent; quite frequently the corresponding drugs are from the classes of analeptics, cardiacs or corticoids. Suitable agents for this purpose are, for example, angiotensine-amide, cardaminol, dobutamine, dopamine, etifelmine,

etilefrine, gepefrine, heptaminol, midodrine, oxedrine, etc., especially norfenefrine;

- at least one substance from the group of anticoagulants. Among other substances, some coumarin-derivatives are suitable for this purpose, as well as heparine and heparinoids, hirudine and related substances, dermatan-sulfate etc.; most frequently used agents of this class are acenocumarin, anisindione, diphenadione, ethyl-biscoumacetate, heparine, hirudine, phenprocoumon, as well as warfarine;

- at least one substance from the class of amtimycotics; well-suited examples of such agents include: amphotericine, bifanozol, buclosamide, chinoline-sulfate chloromidazol, chlorophenesine, chloroquinaldol, clodantoine, cloxiquine, cyclopiroloxamine, dequaliniumchloride, dimazol, fenticlor, flucytosine, griseofulvine, ketoconazol, miconazol, natamycine, sulbentine, tioconazol, tolnaftate, etc.; particularly frequently, amphotericine, clotrimazol or nystatine are likely to be used for this purpose;

- at least one substance from the class of antimyasthenics, such as pyridostigmine-bromide;

- at least one substance which is active against morbus parkinson, such as amantadine, benserazide, benzatropine, biperidene, cycrimine, levodopa, metixene, orphenadrine, phenglutarimide, pridinol, procyclidine, profenamine or trihexyphenidyl;

- at least one substance with an antiphlogistic activity, such as aescine, acetylsalicylic acid, alclofenac, aminophenazone, azapropazone, benzydamine, bumadizone,

chlorothenoxazine, diclofenac, flufenaminic acid, glafenine, ibuprofene, indometacine, kebuzone, mefenam acid, metiazic acid, mesalazine, mofebutazone, naproxene, niflumine acid, salts, such as Na-salt, noramido-pyrinium-methane-sulfonate, orgoteine, oxyphenbutazone, phenylbutazone, propyphenazone, pyridoxine, tolmetine, etc.; very suitable is, for example, ibuprofen; some of the agents commonly used as antiphlogistics also exhibit an antihistaminic or analgetic activity and belong to the classes of corticoids, vasoactiva, opthalmics or otologics;

- at least one substance which is an antipyretic, such as acetylsalicylic acid, alclofenac, aminophenazone, benzydamine, bumadizone, chinine, chlorinethenoxazine, lactylphenetidine, meprob, paracetamol, phenacetine, propyphenazone or salicylamide;

- at least one substance with an antirheumatic activity, such as acetylsalicylic acid, benorilate, chloroquine, diclofenac, fenoprofene, flufenaminic acid, ibuprofene, kebuzone, lactylphenetidine, mefenamic acid, mofebutazone, naproxene, sodiumaurothiomalate, nifenazone, nifluminic acid, D-penicillamine and salicylamide. Edge active substances, carriers and/or agents, with a hypoallergic action, for example from the groups of analgetics, corticoids and glucocorticoids, enzymes or vitamins, etc., are preferred for this purpose, as well as antiphlogistics, such as quinine, nicotinic acid-, nonylic acid-, or salicylic acid-derivatives, meprobamate, etc.;

- at least one antiseptic such as acriflaviniumchloride, cetalkonium-chloride, cetylpyridinium-chloride, chlorohexidine, chloroquinaldol, dequaliniumchloride,

domiphene-bromide, ethacridine, hexetidine, merbromine, nitrofural, oxyquinol, phanquinone, phenazopyridine or phenylmercuriborate, as well as fatty acids with an uneven number of carbon atoms;

- at least one respiratory analeptic or respiration stimulant, such as amiphenazol, ascorbic acid, caffeine, cropropamide, crotethamide, etamivane, ephedrine, fominobene, nicethamide; or aminophenazol and doxaprame, for example;

- at least one broncholytic, such as bamifylline, beclometasone, dexometasone (e.g. in dexometasone-21-isonicotinate), diprophylline, ephinedrine (e.g. in ephinedrinehydrogentartrate), fenoterol, hexoprenaline, ipratropium-bromide, iso-etarine, isoprenaline, orciprenaline, protocylol, proxyphylline, reproterol, salbutamol, terbutaline, tetroquinol, theophyilline, etc.; and biological extracts, for example from anis, eucalyptus, thyme, etc.;

- one cardiotonic, especially aminophylline, benfurodilhemisuccinate, etofylline, heptaminol, protheobromine or proxyphylline;

- at least one substance from the class of chemotherapeutic agents, for example, acediasulfone, acriflavinium-chloride, ambazone, dapsone, dibrompropamidine, furazolidone, hydroxymethyinitrofurantoine, idoxuridine, mafenide and sulfateolamide, mepacrine, metronidazol, nalidixinic acid, nifuratel, nifuroxazide, nifuarazine, nifurtimox, ninorazol, nitrofurantoine, oxolinic acid, pentamidine, phenazopyridine, phthalylsulfatehiazole, pyrimethamine, salazosulfapyridine, sulfacarbamide, sulfacetamide, sulfachloropyridazine, sulfadiazine,

sulfadicramide, sulfadimethoxine, sulfaethidol, sulfafurazol, sulfaguanidine, sulfaguanol, sulfamethizol, sulfamethoxazol and cotrimoxazol, sulfamethoxydiazine, sulfamethoxypyridazine, sulfamoxol, sulfanilamide, sulfaperine, sulfaphenazol, sulfatehiazol, sulfisomidine, tinidazol, trimethoprim, etc.;

- at least one substance from the class of coronary dilatators, such as bamifylline, benziodarone, carbochromes, dilazep, dipyridamol, etafenone, fendiline, hexobendine, imolamine, lidoflazine, nifedipine, oxyfedrine, pentaerythrityltetranitrate, perhexiline, prenylamine, propatylnitrate, racefemine, trolnitrate, verapamil, visnadine, etc.;

- at least one cytostatic, for example, from the group of alkylating agents, antibiotics, platinum compounds, hormones and their inhibitors, interferones, etc.; very frequently used substances of this kind are: aclarubicine, azathioprine, bleomycine, busulfane, calciumfolinate, carboplatinum, carmustine, chloroambucil, cis-platinum, cyclophosphamide, cyt-arabine, daunorubicine, epirubicine, fluorouracil, fosfestrol, hydroxycarbamide, ifosfamide, lomustine, melphalane, mercaptopurine, methotrexate, mitomycine C, mitopodozide, mitramicyne, nimustine, pipobromane, prednimustine, procarbazine, testolactone, theosulfane, thiotepa, tioguanine, triaziquone, trofosfamide, vincristine, vindesine, vinblastine, zorubicine, etc.;

- an intestinal antiseptic, such as broxyquinoline, clioquinol, diodohydroxyquinoline, halquinol, etc.;

- at least one diuretic, such as acetazolamide, aminophylline, bendroflumethiazide, bumetanide, butizide,

chloroazanile, chloromerodrine, chlorothiazide, chlorotalidone, clopamide, clorexolone, cyclopenthiazide, cyclothiazide, etacrynic acid, furosemide, hydrochlorothiazide, hydroflumethiazide, mefruside, methazolamide, paraflutizide, polythiazide, quinethazone, spironolactone, triamterene, trichloromethiazide, xipamide, etc.;

- at least one ganglion blocker, such as gallamintriethiodide, hexamethonium-chloride, mecamylamine, etc.;

- at least one substance for the therapy of arthritis, preferably analgetics or for example allopurinol, benzbromarone, colchicine, benziodarone, probenecide, sulfinpyrazone, tenoxicam, etc.; in very many cases allopurinol;

- at least one glucocorticoid, such as beclomethason, betamethason, clocortolone, cloprednol, cortison, dexamethason (e.g. as a dexamethasonephosphate), fludrocortison, fludroxycortide, flumetason, fluocinolonacetonide, fluocinonide, fluocortolon (e.g. as a fluocortoloncapronate or fluocortolontrimethylacetate), fluorometholon, fluprednidenacetate, hydrocortison (also as a hydrocortison-21-acetate, hydrocortison-21-phosphate, etc.), paramethason, prednisolon (e.g. in the form of methylprednisolon, prednisolon-21-phosphate, prednisolon-21-sulfobenzoate, etc.), prednison, prednyliden, pregnenolon, triamcinolon, triamcinolonacetonide, etc.;

- at least one agent with a putative anti-flew action, such as moroxydine;

- at least one haemostatic, such as adrenalon, ascorbic

acid, butanol, carbazochrome, etamsylate, protamine, samatostatine etc.; thyroidal hormones and vitamins can be employed for this purpose as well;

- at least one hypnotic, from the class of barbiturates, benzodiazepines, bromo-compounds, ureids, etc., for example; quite commonly applied for this purpose are, e.g. acecarbromal, alimemazintartrate allobarbital, amobarbital, aprobarbital, barbital, bromo-isoval, brotizolam, carbromal, chloroalhydrate, chloroalodol, chlorobutanol, clomethiazol, cyclobarbital, diazepam, diphenhydramine, doxylamine, estazolam, ethchlorvynol, ethinamate, etomidate, flurazepam, glutethimide, heptabarb, hexobarbital, lormetazepam, malperol, meclozine, medozine, methaqualon, methyprylon, midazolam, nitrazepam, oxazepam, pentobarbital, phenobarbital, promethazine, propallylonal, pyrithyldion, secbutabarbital, secobarbital, scopolamine, temazepam, triazolam, vinylbital, etc.; various extracts from balm-mint, valerian, and passiflora are also used;

- at least one immunoglobuline, from the IgA, IgE, IgD, IgG, IgM classes or an immunoglobuline fragment, such as a Fab- or Fab2-fragment, or the corresponding variable or hypervariable region, if required in combination with other agents and/or chemically, biochemically or genetically manipulated;

An immunoglobuline can be of the IgA, IgD and IgE, IgG (e.g. Ig G1, Ig G2, Ig G3, Ig G4) or IgM type. In the context of this application, any chemical or biochemical derivative of any immunoglobuline (Ig) is considered useful, for example, an Ig G-gamma chain, an Ig G-F(ab')2 fragment, an Ig G-F(ab) fragment, an Ig G-Fc fragment, an Ig-kappa chain, a light chain of Ig-s (e.g. a kappa and

lambda chain), but also even smaller immunoglobuline fragments, such as the variable or hypervariable regions, or artificial modifications of any of these substances.

- at least one substance with an immunostimulating activity, with an immunosuppressive potency, with a capability to give rise to the production of immunoglobulines or other immunologically active substances (endotoxines, cytokines, lymphokines, prostaglandines, leucotrienes, other immuno modulators or biological messengers), including vaccines. Antibodies against any of these substances can also be used; preferred are immunotransfersomes with or without endotoxines, cytokines, prostaglandines, leucotrienes, with other immunomodulators, immunologically active cellular or molecular fragments, as well as corresponding antagonists, derivatives or precursors; particularly preferred compounds are lipid A and other glycolipids, muraminic acid derivatives, trehalose derivatives, phythaemaglutinines, lectins, polyinosine, polycytidylic acid (poli I:C), dimepranol-4-acetamidobenzoate, erythropoietin, 'granulocyte-macrophage colony stimulating factor' (GM-CSF), interleukine I and II, III and VI, interferon alpha, beta and/or gamma, leucotriene A, B, C, D, E and F, propandiamine, prostaglandine A, B, C, D, E, F, and I (prostacycline), tumor necrosis factor-alpha (TNF-alpha), thromboxan B, as well as immunoglobulines of types IgA, IgE, IgD, IgG, IgM; furthermore, suitable tissue and plant extracts, their chemical, biochemical or biological derivatives or replacements, their parts, such as characteristic peptide chains, etc.; as immunosuppressives, ganciclovir, azathiiprin, cyclosporin, FK 506 etc. are frequently used;

- at least one contraceptive agent, such as medroxyprogesteronacetate, lynesterol, lvonorgestrel, norethisteron, etc.;

- at least one circulation analeptic, such as cafedrin, etamivan, etilefrin, norfenefrin, pholedrin, theodrenalin, etc.;

- at least one drug for the therapy of liver diseases, such as orazamide, silymarin, or tiopromin;

- at least one substance with a light-protective function, such as mexenone;

- at least one antimalaria agent, such as amodiaquin, hydroxychloroquin or mepacrin;

- at least one substance for migraine or schizophrenia treatment, such as certain analeptics, beta-blockers, clonidin, dimetotiazine, ergotamine, lisurid (hydrogen maleate), methysergide, pizotifen, propranolol, proxibarbal, etc. Even more suitble are the serotonine antagonists or the blockers of serotonin receptors, such as 5-HT1, 5-HT2 or 5-HT3; well suited for use according to this invention are also the receptor blockers AH21467 (Glaxo), AH25086 (Glaxo), GR43175 (Glaxo), GR38032 (Glaxo, = ondansetron), 5-hydroxytriptamine, ketanserine, methiothepin, alpha-methyl-5HT, 2-methyl-5HT, etc.;

- at least one mineral corticoid, such as aldosterone, fludrocortison, desoxycortonacetate, corresponding derivatives, etc.;

- at least one morphine antagonist (such as amiphenazol, lealvallorphane, nalorphine) or some substance with

morphine-like properties such as casomorphine, cyclo(leu-gly), dermorphine, met-encephaline, methorphamide (tyr-gly-gly-phe-met-arg-arg-val), morphiceptine, morphine modulating neuropeptide (ala-gly-glu-gly-leu-ser-ser-pro-phe-trp-ser-leu-ala-ala-pro-gln-arg-phe-NH_2) etc.;

- at least one muscle relaxant, which frequently belongs to the groups of competitively or depolarising curare-agents, myotonolytics or analgetics; suitable substances with the desired effect are, among other materials, acetylsalicilic acid, alcuronium-chloride, azapropazon, atracuriumbesilate, baclofen, carisoprodol, quinine derivatives, chloromezanon, chlorophenesincarbamate, chlorozoxazon, dantrolen, decamethoniumbromide, dimethyltubocurariniumchloride, fenyramidol, gallamintriethiodide, guaiphensine, hexafluorenium-bromide, hexacarbacholinbromide, memantin, mephenesin, meprobamate, metamisol, metaxalon, methocarbamol, orphenadrin, paracetamol, phenazon, phenprobamate, suxamethoniumchloride, tetrazepam, tizanidin, tubocurarinchloride, tybamate, etc.;

- at least one narcotic, such as alfentanil, codeine, droperidol, etomidate, fentanil, flunitrazepam, hydroxybutiric acid, ketamine, methohexital, midazolam, thebacon, thiamylal, thiopental, etc., as well as corresponding derivatives;

- at least one substance with a neurotherapeutic activity, such as anaesthetics and vitamins, atropine-derivatives, benfotiamine, choline-derivatives, caffeine, cyanocobolamine, alpha-liponic acid, mepivacaine, phenobarbital, scopolamine, thiaminchloride hydrochloride, etc., and, most notably, procaine;

- at least one neuroleptic, e.g. butyrophenon-derivatives, phenotiazin-derivatives, tricyclic neuroleptics, as well as acetophenazine, benperidol, butaperazine, carfenazine, chloropromazine, chloroprothixen, clopenthixol, clozapine, dixyrazine, droperidol, fluanison, flupentixol, fluphenazine, fluspirilen, haloperidol, homofenazine, levomepromazine, melperon, moperon, oxipertin, pecazine, penfluridol, periciazine, perphenazine, pimozide, pipamperon, piperacetazine, profenamine, promazine, prothipendyl, sulforidazine, thiopropazate, thioproperazine, thioridazine, tiotixen, trifluoperazine, trifluperidol, triflupromazine, etc.; in particular, haloperidol and sulperide are often used for this purpose;

- at least one neurotransmitter or one of its antagonists; preferably, acetylcholine, adrenaline, curare (and, e.g. its antagonist edrophonium-chloride), dopamine, ephedrine, noradrenaline, serotonine, strychnine, vasotonine, tubocurarine, yohimbine, etc. are used;

- at least one opthalmic, in many cases from the groups of anaesthetics, antibiotics, corticoids, eye-tonics, chemotherapeutics, glaucome agents, virustatics, antiallergics, vasodilatators, or vitamins;

- at least one parasympathicomimetic (e.g. bethanechol-chloride, carbachol, demecarium-bromide, distigmin-bromide, pyridostigmin-bromide, scopolamine) or at least one parasympathicolytic (such as benzatropine, methscopolamine-bromide, pilocarpine or tropicamide);

- at least one agent for the therapy of psoriasis and/or neurodermitis; particularly well suited for this purpose are carrier substances with a hypoallergic action or the corresponding edge active compounds, with n-3 (omega 3),

less frequently with n-6 (omega 6), mainly with multiple, often 3-6, double bonds and/or hydroxy, more seldom methyl-, or oxo-side groups; these can also appear as side chains on further agent molecules; side groups on the 15th carbon atom are particularly efficient; as additives, amongst other substances, antimycotics, cytostatics, immunosuppressants or antibiotics can be used;

- at least one agent for the dilatation of the iris (mydriatic), such as atropine, atropinemethonitrate, cyclopentolate, pholedrine, scopolamine or tropicamide;

- at least one substance with a psychostimulating action; well suited for this purpose are, for example, amphetaminil, fencamfamine, fenetylline, meclofenoxate, methamphetamine, methylphenidate, pemoline, phendimetrazine, phenmetrazine, prolintane or viloxazine;

- at least one rhinologic, such as buphenine, cafaminol, carbinoxamide, chlorophenamim, chlorotenoxazine, clemastine, dextromethorpane, etilefrine, naphazoline, norephedrine, oxymetazoline, phenylaprhine, piprinydrinate, pseudoephedrine, salicylamide, tramazoline, triprolidine, xylometazoline, etc.; from biological sources especially the radix gentiane extract;

- at least one somnifacient (such as sleep-inducing peptide (trp-ala-gly-gly-asp-ala-ser-gly-glu)), or a corresponding antagonist (such as bemegride);

- at least one sedative or tranquilizer, as the former, for example, acecarbromal, alimemazine, allobarbital, aprobarbital, benzoctamine, benzodiazepine-derivatives,

bromo-isoval, carbromal, chloropromazine, clomethiazol, diphenyl-methane-derivatives, estazolam, fenetylline, homofenazine, mebutamate, mesoridazine, methylpentynol, methylphenobarbital, molindone, oxomemazine, perazine, phenobarbital, promethazine, prothipendyl, scopolamine, secbutabarbital, trimetozine, etc.; as a tranquilizer, for example, azacyclonol, benactyzin, benzoctamine, benzquinamide, bromo-azepam, chlorodiazepoxide, chlorophenesincarbanate, cloxazolam, diazepam, dipotassium-chloroazepate, doxepine, estazolam, hydroxyzine, lorazepam, medazepam, meprobamate, molindone, oxazepam, phenaglycodol, phenprobamate, prazepam, prochloroperazine, rescinnamine, reserpine or tybamate; drugs, such as distraneurine, hydantoine-derivatives, malonyl uric acid-derivatives (barbiturates), oxazolidine-derivatives, scopolamine, valepotriate, succinimide-derivatives, or hypnotics (e.g. diureides (such as barbiturates)), methaqualon, meprobromate, monoureides (such as carbromal), nitrazepam, or piperidin-dione, can be used for this purpose; amongst other substances, certain thymoleptics, such as librium or tofranil, can be used as antidepressants;

- at least one substance from the class of spasmolytics, e.g. adiphenine, alverine, ambicetamide, aminopromazine, atropine, atropine methonitrate, azintamide, bencyclane, benzarone, bevonium-methylsulfate, bietamiverine, butetamate, butylscopolammoniumbromide, camylofine, carzenide, chlorodiazepoxide, cionium-bromide, cyclandelate, cyclopentolate, dicycloverine, diisopromine, dimoxyline, diphemanil-methylsulfate, ethaverine, ethenzamide, fencarbamide, fenpipramide, fenpivennum-bromide, gefarnate, glycopyrroniumbromide, hexahydroadiphenin, hexocycliummethylsulfate, hymecromon,

isometheptene, isopropamidiodide, levomethadone, mebeverine, metamizon, methscopolamine-bromide, metixen, octatropine-methylbromide, oxazepam, oxybutin, oxyphenonium-bromide, papaverine, paracetamol, pentapiperide, penthienate-methobromide, pethidine, pipenzolate-bromide, piperidolate, pipoxolane, propanthelin-bromide, propylphenazon, propyromazine-bromide, racefemine, scopolamine, sulpiride, tiemonium-iodide, tridihexethyliodide, tropenzilinbromide, tropinbenzilate, trospiumchloride, valethamatbromide, etc.; furthermore, belladonna alkaloids, papaverine and its derivatives, etc.;

- at least one sympathicolytic, e.g. azapetine or phentolamine;

- at least one sympathicomimetic, e.g. bamethane, buphenine, cyclopentamine, dopamine, L-(-)-ephedrine, epinephrine, etilefrine, heptaminol, isoetarine, metaraminol, methamphetamine, methoxamine, norfenefrine, phenylpropanolamine, pholedrine, propylhexedrine, protokylol or synephrine;

- at least one tuberculostatic, such as an antibiotic, p-aminosalicylic acid, capreomycine, cycloserine, dapson, ethambutol, glyconiazide, iproniazide, isoniazide, nicotinamide, protionamide, pyrarinamide, pyrodoxine, terizidone, etc., and, particularly preferred thereof, ethambitol and isoniazide;

- at least one urologic, e.g. a bladder tension modifying agent (such as cholinecitrate, distigminebromide, yohimbine), a corresponding antiinfection agents (antibiotics, chemotherapeutics, or nitrofurantoid-, chinolone-, or sulfonamide-derivative); furthermore,

adipinic acid, methionine, methenamine-derivatives, etc.;

- at least one substance with a vasoconstricting action; often, adrenalone, epinephrine, felypressine, methoxamine, naphazoline, oxymetazoline, tetryzoline, tramazoline or xylometazoline are used for this purpose;

- at least one substance which is a vasodilatator, such as e.g. azapetine, banethane, bencyclane, benfurodil-hemisuccinate, buphenine, butalamine, cinnarizine, diprophylline, hexyltheobromine, ifenprodil, isoxsuprine, moxisylyte, naftidrofuryl, nicotinylalcohol, papaverine, phenoxybenzamine, piribedil, primaperone, tolazoline, trimetazidine, vincamine or xantinol-nicotinate;

- at least one veins agent, e.g. aescine, benzarone, calcium-dobesilate, dihydroergotaminemesilate, diosmine, hyydroxyethylrutoside, pignogenol, rutoside-aesinate, tribenoside, troxerutine, etc.;

- at least one virustatic, e.g. one immunostimulating agent, and/or an additional drug, such as as moroxydine or tromantadine, which may stimulate action of the immunostimulator;

- one agent for the treatment of wounds; for example, dexpanthenol, growth stimulating factors, enzymes or hormones, especially in combination with carriers which contain essential substances; povidon-iodide, fatty acids which are not straight, cetylpyridiniumchloride, chinoline-derivatives of known antibiotics and analgetics are useful;

- at least one substance with a toxic action or a toxin; common toxins from plant or microbial sources in

particular 15-acetoxyscirpenol, 3-acetyldeoxynivalenol, 3-alpha-acetyldiacetoxyscirpenol, acetyl T-2 toxin, aflatoxicol I, aflatoxicol II, aflatoxin B1, aflatoxin B2, aflatoxin B2-alpha, aflatoxin G1, aflatoxin G2, aflatoxin G2-alpha, aflatoxin M1, aflatoxin M2, aflatoxin P1, aflatoxin Q1, alternariol-monomethyl ether, aurovertin B, botulinum toxin D, cholera toxin, citreoviridin, citrinin, cyclopiazonic acid, cytochalasin A, cytochalasin B, cytochalasin C, cyrochalasin D, cytochalasin, cytochalasin H, cytochalasin J, deoxynivalenol, diacetoxyscirpenol, 4,15-diacetylverrucarol, dihydrocytochalasin B, enterotoxin STA, fusarenon X, iso T-2 toxin, O- methylsterigmatocystin, moniliformin, monoacetoxyscirpenol, neosolaniol, ochratoxin A, patulin, penicilinic acid, pertussis toxin, picrotoxin, PR-toxin, prymnesin, radicinin, roridin A, rubratoxin B, scirpentriol, secalonic acid D, staphylococcalenterotoxin B, sterigmatocystin, streptolysin O, streptolysin S, tentoxin, tetrahydrodeoxyaflatoxin B1, toxin A, toxin II, HT-2 toxin, T-2-tetraol, T-2 toxin, trichothecin, trichothecolon, T-2 triol, verrucarin A, verrucarol, vomitoxin, zearalenol and zearalenon.

- at least one substance which affects growth in humans or animals, such as basic fibroblast growth factor (BFGF), endothelial cell growth factor (ECGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), insulin, insulin-like growth factor I (LGF I), insulin-like growth factor II (LGF II), nerves-growth factor-beta (NGF-beta), nerves growth-factor 2,5s (NGF 2,5s), nerves growth-factor 7s (NGF 7s), platelet-derived growth factor (PDGF), etc.;

- a carrier and/or agent which creates a protective layer on and/or in a barrier, such as skin, against poison,

light UV-, gamma- or other radiation; against detrimental biological agents such as viruses, bacteria, toxins, etc.; carrier components and/or agents can hamper the detrimental action by chemical, biochemical, or biological means or else may prevent or diminish the penetration of such adversary agents;

- at least one fungicide, herbicide, pesticide, or insecticide;

- at least one plant hormone, e.g. abscisic acid, abscisic acid-methylester, 3-acetyl-4-thiazolidine-carboxyl acid, 1-allyl-1-(3,7-dimethyloctyl)-piperidinium bromide, 6-benzylaminopurine, 6-benzylaminopurine 9-(beta-glucoside), butanedio acid mono(2,2-dimethyl hydrazide), chlorocholine chloride, 2-chloroethyl-tris-(2'-methoxyethoxy)silane, 2-(o-chlorineophenoxy)-2-methylpropionic acid, 2-(p-chlorophenoxy)-2-methylpropionic acid, 2-(o-chlorophenoxyipropionic acid, 2-(m-chlorophenoxy)propionic acid, clofibrinic acid, colchicine, o-coumarinic acid, p-coumarinic acid, cycloheximide, alpha,beta-dichloroisobutiric acid, 2-(2,4-dichlorophenoxy)propanoic acid, 2,3-dihydro-5,6-diphenyl 1,4-oxathiine, dihydrozeatine, 6-(gamma,gamma-dimethylallylamino)purino riboside, 3-(2-[3,5-dimethyl-2-oxocyclohexyl-2-hydroxyethyl])-glutarimide, trans-2-dodecenedioic acid, ethyl-8-chloro-1-indazol-3-yl-acetate, N6-furfuryladenosine, 6-furfurylamino-purineriboside, gibberellic acid methylester, gibberellin A3-acetate, gibberellin A1 methylester, gibberellin A4 methylester, gibberellin A5 methylester, gibberellin A7 methylester, gibberellin A9 methylester, gibberellin A3 methylester 3,13-diacetate gibberinic acid, allo-gibberinic acid, gibberinic acid methylester, glyoxim, 22(s),23(s)-homobrassinolide, 9-hydroxyfluorene 9-

carboxylate, indol-3-acetic acid, indol-3-acetic acid ethylester, indol-3-propanoic acid, N6-(2-isopentenyl)adenine, N6-(2-isopentenyl)adenosine, 2-isopropyl-4-dimethylamino-5-methylphenyl-1-piperidine-carboxylat methylchloride, kinetinglucoside, kinetinriboside, melissylalcohol, 1-methyladenine, methyl 2-chloro-9-hydroxy-fluorene-9-carboxylate, methyl 3,6-dichloro-o-anisate, 6-methylmercaptopurine, 1-naphthylacetamide, nonanoic acid methylester, 6-piperidino-1-purine, n-triacontanol, (-)-xanthoxine, zeatine glucosides, etc.;

- at least one pheromone or one pheromone-like substance, such as (-)-bornyl acetate, trans-5-decenol, cis-5-decenyl acetate, trans-5-decenyl acetate, 2,6-dichlorophenol, 1,7-dioxaspiro[5.5]undecane, trans-8,trans-10-dodecadienol ([E,E]-8,10-DDDOL), trans-7, cis-9-dodecadienyl acetate ([E,Z]-7,9-DDDA), trans-8, trans-10-dodecadienyl acetate ([E,E]-8,10-DDDA), cis-7-dodecen-1-ol (Z-7-DDOL), trans-10-dodecenol, cis-7-dodecenyl acetate (Z-7-DDA), cis-8-dodecenyl acetate, trans-8-dodecenyl acetate, 11-dodecenyl acetate, cis-7,8-epoxy-2-methyl-octadecane, cis-9-heneicosene, cis-7,cis-11-hexadecadienylacetate ([Z,Z]-7,11-HDDA), cis-7,trans-11- hexadecadienyl acetate ([Z,E)-7,11-HDDA), cis-9-hexadecenal (Z-9-HDAL), cis-11-hexadecenal (Z-11-HDAL), cis-11-hexadecenol (Z-11-HDOL), cis-11-hexadecenyl acetate (Z-11-HDA), trans-2-hexenyl acetate, cis-7-tetradecenal (Z-7-TDAL), cis-9-tetradecenol (Myristoleyl alcohol; Z-9-TDOL), cis-7-tetradecenol (Z-7-TDOL), cis-11-tetradecenol, cis-7-tetradecenyl acetate (Z-7-TDA), cis-9-tetradecenyl acetate (Myristoleyl acetate; Z-9-TDA), cis-11-tetradecenyl acetate (Z-11-TDA), trans-11-tetradecenyl acetate (E-11-TDA), cis-9-tetradecenyl formate

(Myristoleyl formate; Z-9-TDF), isoamyl acetate (acetic acid 3-methylbutyl ester), 2-methyl-3-buten-2-ol, 3-methyl-2-cyclohexen-1-ol, cis-14-methyl-8-hexadecenal, cis-2-methyl-7-octadecene, 4-methylpyrrole-2-carboxylic acid methyl ester (Methyl 4-methylpyrrole 2-carboxylate) cis-13-octadecenal 13-octadecyn-1-ol, 2-(phenyl)ethyl propionate (phenylethanol propanoate), propyl cyclohexylacetate, cis-9,trans-11-tetradecadienol ([Z,E]-9,11-TDDOL), cis-9,trans-11-tetradecadienyl acetate ([Z,E]-9,11-TDDA), cis-9,trans-12-tetradecadienyl acetate ([Z,E]-9,12-TDDA), trichloroacetic acid esters, cis-9-tricosene, undecanal, etc.;

- at least one pigment or one colouring substance;

- at least one carbohydrate;

A carbohydrate, normally, has a basic formula $C_x(H_2O)_y$, e.g. in sugar, starch, cellulose, and, moreover, can be derivatised in many different ways.

A monomeric carbohydrate residue is, for example, a natural monosaccharide residue, which in many cases is an adduct of a pentose or a hexose in aldose or ketose form which, in principle, can adopt L- or D-configurations. Owing to the space constraints and due to their greater biological relevance, only the latter will be referred to in the following.

An aldose with five carbon atoms (aldo-pentose, or simply pentose) is for example D-arabinose, D-lyxose, D-ribose or D-xylose.

A ketose with five carbon atoms (keto-pentose) is e.g. D-ribulose or D-xylulose.

An aldose with six carbon atoms (aldo-hexose, or simply hexose) is e.g. D-allose, D-altrose, D-galactose, D-glucose, D-mannose or D-talose. A ketose with six carbon atoms (or simply keto-hexose) is e.g. D-fructose, D-psicose, D-sorbose or D-tagatose.

A hexose, very frequently, exists in a cyclic form, as a pyranose (aldose), for example; alpha- or beta-D-glucopyranose are two typical examples for this. Another type of hexose is furanose, e.g. in an alpha- or beta-D-fructose. The pyranosyl residue is particularly preferably conjugated to a hydroxy group, the latter then being located in 1- or 6-positions; the furanosyl residue is preferably conjugated to the corresponding groups in positions 1- or 5-.

A carbohydrate residue, moreover, can be a natural disaccharide residue, e.g. a disaccharide residue consisting of two hexoses. Such a disaccharide residue arises, for example, through condensation of two aldoses, e.g. D-galactose or D-glucose, or one aldose, e.g. D-glucose and one ketose, e.g. fructose; disaccharides formed from two aldoses, such as lactose or maltose, are preferably conjugated to the phosphatidyl group through the hydroxy group, which is located in position 6- of the corresponding pyranosyl residue. A disaccharide formed from an aldose and a ketose, such as saccharose, is preferably conjugated through a hydroxyl-group in position 6- of the pyranosyl residue or in position 1- of the furanosyl residue.

A carbohydrate residue, moreover, is any derivatised mono-, di- or oligosaccharide residue, in which, for example, an aldehyde group and/or one or two terminal

hydroxy groups are oxidized to carboxy groups, e.g. in a D-glucar-, D-glucon- or D-glucoronic acid residue, all such residues being normally in the form of cyclic lactone residues. The aldehyde- or keto-groups in a derivatised mono- or disaccharide residue, moreover, can be reduced to hydroxy groups, e.g. in inositol, sorbitol or D-mannitol. Furthermore, individual hydroxy groups can be replaced by hydrogen atoms, e.g. in desoxysugars, such as 2-desoxy-D-ribose, L-fucose or L-rhamnose, or through amino groups, e.g. in aminosugars, such as D-galactosamine or D-glucosamine.

A carbohydrate can result from a cleaving action, starting with one of the mentioned mono- or disaccharides, by a strong oxidation agent, such as periodic acid. Amongst the biologically most important or most active carbohydrates are e.g. 2-acetamido-N-(epsilon-amino-caproyl)-2-deoxy-beta-gluccopyrano-sylamine, 2-acetamido-1-amino-1,2-dideoxy-beta-glucopyranose, 2-acetamido-1-beta-(aspartamido)-1,2-dideoxyglucose, 2-acetamido-4,6-o-benzyliden-2-deoxy-beta-glucopyranose, 2-acetamido-2-deoxyallose, 3-acetamido-3-deoxyallose, 2-acetamido-2-deoxy-3-o-(beta-galactopyranosyl)-galactopyranose, 2-acetamido-2-deoxy-4-o-([4-o-beta-galactopyranosyl-beta-galactopyranosyl]-beta-galactopyranosyl)-glucopyranose, 2-acetamido-2-deoxy-3-o-(beta-galactopyranosyl)-alpha-glucopyranose, 6-o-(2-acetamido-2-deoxy-4-o-[beta-galactopyranosyl]-beta-glucopyranosyl)-galactopyranose, 4-o-acetamido-2-deoxy-6-o-(beta-galacto-4-o-(6-o-[2-acetamido-2-deoxy-beta-glucopyranosyl]-beta-galactopyranosyl) glucopyranose, 2-acetamido-2-deoxygalactose, 2-acetamido-2-deoxyglucose, 3-acetamido-3-deoxyglucose pyranose, 6-o-(2-acetamido-2-deoxy-beta-glucopyranosyl)-galactopyranose, 2-acetamido-2-deoxy-1-thio-beta-glucopyranose 3,4,6-

triacetate, acetopyruvic acid, N-acetylchondrosamine, N-acetylgalactosamine, N-acetylglucosamine, N-acetyl-alpha-glucosamine 1-phosphate, N-acetylglucosamine 6-phosphate, N-acetylglucosamine 3-sulfate, N-acetylglucosamine 6-sulfate, N-acetylheparine, N-acetyllactosamine, N-acetyl-beta- mannosamine, N-acetylneuraminic acid, N-acetyl-neuramine-lactose, 1-o-acetyl-2,3,5-tri-o-benzoyl-beta-ribofuranose, trans-aconic acid, adenine-9-beta-arabino-furanoside, adenosine 5'-diphospho-glucose, adenosine 5'-diphosphomannose, adonite, adonitol, adonose, agar, algin, alginic acid, beta-allose, alpha glycerophosphate, alpha ketoglutaric acid, altrose, (-)-altrose, p-amino-benzyl-1-thio-2-acetamido-2-deoxy-beta-glucopyranoside, N-epsilon-aminocaproyl-beta-fucopyranosylamine, N-epsilon-aminocaproyl-alpha-galactopyranosylamine, 2-amino-2-deoxygalactopyranose, 6-amino-6-deoxygluco-pyranose, 1-amino-1-deoxy-beta-glucose, 6-aminohexyl-N-acetyl-beta-thioglucosaminide, 6-aminohexyl-1-thio-beta-galactopyranoside, 5-aminoimidazole-4-carboxamidoxime-1-beta-ribofuranosyl 3':5'-cyclo-monophosphate, delta-aminolevulinic acid, p-aminophenyl-2-acetamido-2-deoxy-beta-glucopyranoside, p-aminophenyl-2-acetamido-2-deoxy-1-thio-beta-glucopyranoside, p-aminophenyl-alpha-fucopyranoside, p-aminophenyl-alpha-galactopyranoside, p-aminophenyl-beta-galactopyranoside, p-aminophenyl-alpha-glucopyranoside, p-aminophenyl-beta-glucopyranoside, c-aminophenyl-beta-glucuronide, p-aminophenyl-1-thio-beta-glucuronide, p-aminophenyl-beta-lactopyranoside, p-aminophenyl-alpha-mannopyranoside, p-aminophenyl-beta-thiofucopyranoside, p-aminophenyl-1-thio-beta-galactopyranoside, p-aminophenyl-1-thio-beta-glucopyranoside, p-minophenyl-1-thio-beta-xylopyranoside, p-aminophenyl-beta-xylopyranoside, 5-amino-1-(beta-ribofuranosyl)imidazole 4-carboxamide, amygdaline, n-amyl beta-glucopyranoside, amylopectine, amylose, apigenine 7-

o-hesperidoside, arabinitol, arabinocytidine, 9-beta-arabinofuranosyladenine, 1-beta-arabinofuranosylcytosin, arabinose, arabinose 5-phosphate, arabinosylcytosine, arabite, arabitol, arbutine, atp-ribose, atractyloside, aurothioglucose, n-butyl 4-o-beta-galactopyranosyl-beta-glucopyranoside, calcium gluconate, calcium heptagluconate, carboxyatractyloside, carboxymethylamylose, carboxymethylcellulose, carboxyethylthioethyl-2-acetamido-2-deoxy-4-o-beta-galactopyransol-beta-glucopyranoside, carboxyethylthioethyl 4-o-(4-o-[6-o-alpha-glucopyranosyl-alpha-glucopyranosyl]-alpha-glucopyranosyl)-beta-glucopyranoside, 4-o-(4-o-[6-o-beta-D-galactopyranosyl-beta-D-galactopyranosyl]-D-glucopyranose, carrageenan, D(+)cellobiose, D(+)cellopentaose, D(+)cellotetraose, D(+)cellotriose, cellulose, cellulose caprate, cellulose carbonate, chitin, chitobiose, chitosan, chitotriose, alpha-chloroalose, beta-chloroalose, 6-chloro-6-deoxy-alpha-glucopyranose, chondroitin sulfate, chondrosamine, chondrosine, chrysophanic acid, colominic acid, convallatoxin, alpha-cyclodextrine, beta-cyclodextrine, cytidine 5'-diphosphoglucose, cytosine 1-beta-arabinofuranoside, daunosamine, n-decyl-beta-glucopyranoside, 5-deoxyarabinose, 2-deoxy-2-fluoroglucose, 3-deoxy-3-fluoroglucose, 4-deoxy-4-fluoroglucose, 6-deoxygalacto pyranose, 2-deoxygalactose, 1-deoxyglucohex-1-eno-pyranose tetrabenzoat, 2-deoxyglucose, 6-deoxyglucose, 2-deoxyglucose 6-phosphate, 1-deoxymannojerimycin, 6-deoxymannose, 1-deoxy-1-morpholinofructose, 1-deoxy-1-nitroalutol, 1-deoxy-1-nitroaltitol, 1-deoxy-1-nitrogalactitol, 1-deoxy-1-nitromannitol, 1-deoxy-1-nitrosorbitol, 1-deoxy-1-nitrotalitol, deoxynojirimycine, 3-deoxy-erythro-pentose, 2-deoxy-6-phosphogluconic acid, 2-deoxyribose, 3-deoxyribose, 2-deoxy-alpha-ribose 1-

phosphate, 2-deoxyribose 5-phosphate, 5-deoxyxylofuranose, dextran, dextransulfate, dextrine, dextrose, diacetonefructose, diacetonemannitol, 3,4-di-o-acetyl-6-deoxyglucal, di-o-acetylrhamnal, 2,3-diamino-2,3-dideoxy-alpha-glucose, 6,9-diamino-2-ethoxyacridine lactate, 1,3:4,6-di-o-benzylidene mannitol, 6,6'-dideoxy-6,6'-difluorotrehalose, digalactosyl diglyceride, digalacturonic acid, (+)digitoxose, 6,7-dihydrocoumarin-9-glucoside, dihydroxyacetone, dihydroxyacetone phosphate, dihydroxyfumaric acid, dihydroxymalic acid, dihydroxytartaric acid, dihydrozeatinriboside, 2,3-diphosphoglycerolic acid, dithioerythritol, dithiothreitol, n-dodecyl beta-glucopyranoside, n-dodecyl beta-maltoside, dulcitol, elemi-gum, endotoxin, epifucose, erythritol, erythro-pentulose, erythrose, erythrose 4-phosphate, erythrulose, esculin, 17-beta-estradiol-3-glucuronide 17-sulfate, estriole glucuronide, estron beta-glucuronide, ethodin, ethyl 4-o-beta-D-galactopyranosyl)-beta-D-glucopyranoside, ethyl2-acetamido-4-o-(2-acetamido-2-deoxy-beta-glucopyranosyl)-6-o-(alpha -fucopyranosyl)-2-deoxy-beta-glucopyranoside, ethyl2-acetamido-2-deoxy-4-o-(4-o-alpha-galactopyranosyl-beta-galactopyranosyl)-beta-glucopyranoside, ethyl cellulose ethylene glycol chitin, ethyl 4-o-(4-o-alpha-galacto-pyranosyl-beta-galactopyranosyl)-beta-glucopyranoside, ethyl 4-o-beta-galactopyranosyl-beta-glucopyranoside, ethyl pyruvate, ethyl beta-thioglucoside, etiocholane-3alpha-ol-17-on glucuronide, ficoll, 6-fluoro-6-deoxyglucose, franguloside, fraxin, fructosazine, beta-(-)fructose, fructose-1,6- diphosphate, fructose-2,6-diphosphate, fructose-1-phosphate, fructose-6-phosphate, fucoidan, fucose, alpha -(-)-fucose-1-phosphate, fucosylamine, 2'-fucosyllactose, 3-fucosyllactose, fumaric acid, galactal,

galactitol, galactopyranosylamine, 3-o-beta-galactopyranosyl-arabinose, 4-o-beta-galactopyranosyl-fructofuranose, 4-o-(4-o-beta-galactopyranosyl beta-galactopyranosyl)-glucopyranose, 4-o-alpha-galactopyranosyl- galactopyranose, 6-o-beta-galactopyranosylgalactose, 4-o-(beta-galactopyranosyl)-alpha-mannopyranose, alpha-galactopyranosyl 1-phosphate, galactopyranosyl-beta-thio-galactopyranoside, (+)galactosamine, alpha-galactosamine 1-phosphate, alpha-galactose 1-phosphate, galactose 6-phosphate, galactose 6-sulfate, 6-(alpha-galactosido)glucose, galacturonic acid, beta-gentiobiose, glucan, glucitol, glucoheptonic acid, glucoheptose, glucoheptulose, gluconate 6-phosphate, gluconic acid, 1-o-alpha-glucopyranosyl-beta-fructofuranoside, 6-o-alpha-glucopyranosylfructose, 1-o-alpha-glucopyranosyl-alpha-glucopyranoside, 4-o-beta-glucopyranosylglucopyranose, 4-o-(4-o-[6-o-alpha-glucopyranosyl-alpha-glucopyranosyl]-alpha-glucopyranosyl) glucopyranose, (+)glucosamine, alpha-glucosamine 6-2,3-disulfate, alpha-glucosamine 1-phosphate, glucosamine 6-phosphate, glucosamine 2-sulfate, alpha-glucosamine 3-sulfate, glucosamine 6-sulfate, glucosaminic acid, glucose, alpha-glucose 1,6-diphosphate, glucose 1-phosphate, glucose 6-phosphate, glucose 6-sulfate, glucuronamide, glucuronic acid, alpha-glucuronic acid 1-phosphate, glyceraldehyde, glyceraldehyde 3-phosphate, glycerate 2,3-diphosphate, glycerate 3-phosphate, glyceralic acid, alpha-glycerophosphate, beta-glycerophosphate, glycogen, glycolaldehyde, glycol chitosan, n-glycolylneuraminic acid, glycyric acid, glyoxylic acid, guanosine, 5'-diphosphoglucose, gulose, gums (accroides, agar, arab, carrageenan, damar, elemi, ghatti, guaiac, guar, karaya, locust bonne, mast, pontianac, storax, tragacanth, xanthan), heparin and heparin-like substances

(mesoglycan, sulodexide, etc.), heptakis (2,3,6-tri-o-methyl)-beta-cyclodextrin, heptanoyl-N-methylglucamide, n-heptyl beta-glucopyranoside, hesperidin, n-hexyl-beta-glucopyranoside, hyaluronic acid, 16-alpha-hydroxyestronglucuronide, 16-beta-hydroxyestron glucuronide, hydroxyethyl starch, hydroxypropylmethyl-cellulose, 8-hydroxyquinolin-beta-glucopyranoside, 8- hydroxyquinolin glucuronide, idose, (-)-idose, indole-3- lactic acid, indoxyl-beta-glucoside, epi-inositol, myo-inositol, myo-inositol bisphosphate, myo-inositol-1,2-cyl phosphate, scyllo-inositol, inositolhexaphosphate, inositolhexasulfate, myo-insoitol 2-monophosphate, myo-inositol trisphosphate, (q)-epi-inosose-2, scyllo-inosose, inulin, isomaltose, isomaltotriose, isosorbid dinitrate, 11-ketoandrosterone beta-glucuronide, 2-ketogluconic acid, 5-ketogluconic acid, alpha-ketopropionic acid, lactal, lactic acid, lactitol, lactobionic acid, lacto-N-tetraose, lactose, alpha-lactose 1-phosphate, lactulose, laminaribiose, laminnarine, levoglucosan, beta-levulose, lichenan, linamarine, lipopolysaccharides, lithiumlactate, lividomycine A, lyxose, lyxosylamine, maltitol, maltoheptaose, maltohexaose, maltooligosaccharide, maltopentaose, maltose, alpha-(+)maltose 1-phosphate, maltotetraose, maltotriose, malvidine-3,5-diglucoside, mandelonitril beta-glucoside, mandelonitril glucuronic acid, mannan, mannit, mannitol, mannitol 1-phosphate, alpha-mannoheptitol, mannoheptulose, 3-o-alpha-mannopyranosyl-mannopyranose, alpha(+)mannopyranosyl-1-phosphate, mannosamine, mannosan, mannose, A(+)mannose 1-phosphate, mannose 6-phosphate, (+)melezitose, A(+)melibiose, mentholglucuronic acid, 2-(3'-methoxyphenyl)-N-acetylneuraminic acid, methyl 3-o-(2-acetamido-2-deoxy-beta-galactopyranosyl)-alpha-

galactopyranoside, methyl 4-o-(3-o-[2-acetamido-2-deoxy-4-o-beta-galactopyranosyl beta-glucopyranosyl]-beta-galactopyranosyl)-beta-glucopyranoside, methyl 2-acetamido-2-deoxy-beta-glucopyranoside, methyl3-o-(2-acetamido-2-deoxy-beta-glucopyranosyl)-beta-galactopyranoside, methyl6-o-(2-acetamido)-2-deoxy-beta-glucopyranosyl)-alpha-mannopyranoside, methyl acosaminide, methyl alpha-altropyranoside, methyl3-amino-3-deoxy-alpha-mannopyranoside, methyl beta-arabinopyranoside, methyl 4,6-o-benzylidene-2,3-di-o-toluenesulfonyl-alpha-galactopyranoside, methyl 4,6-o-benzylidene-2,3-di-o-p-toluenesulfonyl-alpha-gluco-pyranoside, methyl cellulose, methyl alpha-daunosaminide, methyl6-deoxy-alpha-galactopyranoside, methyl 6-deoxy-beta-galactopyranoside, methyl 6-deoxy-alpha-glucopyranoside, methyl 6-deoxy-beta-glucopyranoside, methyl 3,6-di-o-(alpha-mannopyranosyl)-alpha-mannopyranoside, 1-o-methyl-alpha-galactopyranoside, 1-o-methyl-beta-galactopyranoside, methyl 3-o-alpha-galactopyranosyl-alpha-galactopyranoside, methyl-3-o-beta-galactopyranosyl-beta-galactopyranoside, 4-o-(2-o-methyl-beta-galactopyranosyl) glucopyranose, methyl 4-o-beta-galactopyranosyl-beta-glucopyranoside, methyl-4-o-(beta-galactopyranosyl-alpha-mannopyranoside, 5-5-methylgalacto pyranose, methylgalactoside, n-methylglucamine, 3-o-methyl-alpha-glucopyranose, 1-o-methyl-alpha-glucopyranoside, 1-o-methyl-beta-glucopyranoside, alpha-methyl glucoside, beta-methyl glucoside, methyl glycol chitosan, methyl-alpha-mannopyranoside, methyl-2-o-alpha-mannopyranosyl-alpha-mannopyranoside, methyl 3-o-alpha-mannopyranosyl-alpha-mannopyranoside, methyl-4-o-alpha-mannopyranosyl-alpha-mannopyranoside, methyl 6-o-alpha-mannopyranosyl-alpha-mannopyranoside, methyl alpha-rhamnopyranoside, methyl alpha-ribofuranoside, methyl beta-ribofuranoside,

methylbeta-thiogalactoside, methyl 2,3,5-tri-o-benzoyl-alpha-arabinofuranoside, 4-methylumbelliferyl2-acetamido-4,6-o-benzylidene-2-deoxy-beta-glucopyranoside, 4-methylumbelliferyl N-acetyl-beta-galactosaminide, 4-methylumbelliferyl N-acetyl-alpha-glucosaminide, 4-methylumbelliferyl-N-acetyl-beta-glucosaminide, 4-methylumbelliferyl-alpha-arabinofuranoside, 4-methylum-belliferyl-alpha-arabinopyranoside, 4-methylum-belliferyl-beta-cellobioside, 4-methylumbelliferyl-beta-n,n'-diacetylchitobioside, 4-methylumbelliferyl alpha-fucoside, 4-methylumbelliferyl beta-fucoside, 4-methylumbelliferyl alpha-galactopyranoside, 4-methylumbelliferyl beta-galactopyranoside, 4-methylumbelliferyl alpha-galactoside, 4-methylumbelliferyl beta -glucopyranoside, 4-methylumbelliferyl alpha-glucoside, 4-methylumbelliferyl beta-glucoside, 4-methylumbelliferyl beta-glucuronide, 4-methylumbelliferyl beta-mannopyranoside, 4-methylumbelliferylbeta-n,n',n''-triacetylchitotriose, 4-methylumbelliferyl2,3,5-tri-o-benzyl-alpha-arabinofuranoside, 4-methylumbelliferyl beta-xyloside, methyl beta-xylopyranoside, 2-o-methylxylose, alpha-methylxyloside, beta-methylxyloside, metrizamide, 2'-monophosphoadenosine 5'-diphosphoribose, 2'-monophosphoinosine 5'-diphosphoribose, mucine, muraminic acid, naringine, sodium lactate, sodium polypectate, sodium pyruvate, neoagarobiose, neoagarohexaitol, neoagarohexaose, neoagarotetraose, beta-neocarrabiose, neocarrabiose 4/1-sulfate, neocarrahexaose(2/4,4/1,4/3,4/5)-tetrasulfate, neocarratetraose(4/1,4/3)-disulfate, neocarratetraose(4/1)-sulfate, neohesperidin, dihydrochalcon, neohesperidose, neuraminic acid, neuraminic acid beta-methylglycoside, neuramine-lactose, nigeran, nigerantetrasaccharide, nigerose, n-nonyl glucoside, n-nonylbeta-glucopyranoside, octadecylthio-ethyl 4-o-alpha-galactopyranosyl-beta-galactopyranoside,

octadecylthioethyl 4-o-(4-o-[6-o-alpha-glucopyranosyl-alpha-glucopyranosyl]-alpha-glucopyranosyl)-beta-glucopyranoside, octanoyl n-methylglucamide, n-octyl alpha-glucopyranoside, n-octyl-beta-glucopyranoside, oxidised starch, pachyman, palatinose, panose, pentaerythritol, pentaerythritol diformal, 1,2,3,4,5-pentahydroxy, capronic acid, pentosanpolysulfate, perseitol, phenolphthalein glucuronic acid, phenolphthalein mono-beta-glucosiduron phenyl 2-acetamido-2-deoxy-alpha-galactopyranoside, phenyl2-acetamido-2-deoxy-alpha- glucopyranoside, alpha-phenyl-N-acetyl-glucosaminide, beta-phenyl N-acetyl-glucosaminide, phenylethyl beta- galactoside, phenyl beta-galactopyranoside, phenyl beta-galactoside, phenyl alpha-glucopyranoside, phenyl beta-gluco-pyranoside, phenyl alpha-glucoside, phenyl beta-glucoside, phenyl beta-glucuronide, beta-phenyllactic acid, phenyl alpha-mannopyranoside, beta-phenylpyruvic acid, phenyl beta-thiogalactopyranoside, phenyl beta-thiogalactoside, phospho(enol)pyruvate, (+)2-phosphoglyceric acid, (-)3-phosphoglyceric acid, phosphohydroxypyruvic acid, 5-phosphorylribose 1-pyrophosphate, phytic acid, poly-N-acetylglucosamine, polygalacturonic acid, polygalacturonic acid methyl ester, polypectate, sodium, polysaccharide, 5beta-pregnane-3alpha,2oalpha-diol glucuronide, n-propyl4-o-beta-galactopyranosyl-beta-glucopyranoside, prunasine, psicose, pullulan, quinolyl-8beta-glucuronic acid, (+)raffinose, alpha-rhamnose, rhapontine, ribitol, ribonolacton, ribose, D-2-ribose, alpha-ribose 1-phosphate, ribose 2-phosphate, ribose 3-phosphate, ribose 5-phosphate, ribulose, ribulose-1,5-diphosphate, ribulose 6-phosphate, saccharic acid, saccharolactic acid, saccharose, salicin, sarcolactic acid, schardingers-alpha-dextrine, schardingers-beta-dextrine,

sedoheptulosan, sedoheptulose 1,7-diphosphate, sialic acid, sialyllactose, sinigrine, sorbitol, sorbitol 6-phosphate, (+)-sorbose, (-)sorbose, stachyose, starch, storax, styrax, sucrose, sucrose monocaprate, tagatose, alpha-talose, (-)-talose, tartaric acid, testosterone-beta-glucuronide, 2,3,4,6-tetra-o- methyl-glucopyranose, thiodiglucoside, 1-thio-beta- galactopyranose, beta-thioglucose, 5-thioglucose, 5- thioglucose 6-phosphate, threitol, threose, (+)threose, (-)threose, thymidine 5'-diphosphoglucose, thymin 1-beta- arabinofuranoside, tragacanth, (+)trehalose, trifluorothymin, deoxyriboside, 3,3',5-trihydroxy-4'- methoxy-stilbene-3-o-beta-glucoside, trimethylsilyl(+)arabinose, trimethylsilyldulcitol, trimethylsilyl-beta (-) fructose, trimethylsilyl(+) galactose, trimethylsilyl-alpha-(+)-glucose, trimethylsilyl(+) mannitol, trimethylsilyl(+]rhamnose, trimethylsilyl(-) sorbitol, trimethylsilyl(+)xylose, rac-1-o-tritylglycerol, (+)turanose, n-undecyl beta-glucopyranoside, uracil beta-arabinofuranoside, uridine 5'-diphospho-N-acetylglucosamine, uridine 5'-diphosphogalactose, uridine 5'-diphosphoglucose, uridine 5'-diphospho-glucuronic acid, uridine 5'-diphosphomannose, uridine 5'-diphosphoxylose, vancomycine, xanthan gum, xylane, xylite, xylitol, xylobiose, alpha-xylopyranosyl 1-phosphate, xylose, alpha-xylose 1-phosphate, xylose 5-phosphate, xylotriose, xylulose, xylulose 5-phosphate, yacca, zeatine riboside, zinclactate, zymosan A, etc.

Denotations desoxyribonucleic-(DNA) and ribonucleic acid (RNA) have their common meaning; preferably such DNA or RNA forms, or their antagonists, are used which have a particularly strong biological action.

- at least one nucleotide, peptide, protein or a related compound;

Nucleotides, which can be effectively transported with the aid of transfersomes, encompass adenine, adenosine, adenosine-3',5'-cyclic monophosphate, N6,O2'-dibutyryl, adenosine-3',5'-cyclic monophosphate, N6,O2'-dioctanoyl, adenosine, n6-cyclohexyl, salts of adenosine-5'-diphosphate, adenosine-5'-monophosphoric acid, adenosine-5'-o-(3-thiotriphosphate), salts of adenosine-5'-triphosphate, 9-beta-D-arabinoturanosyladenine, 1-beta-D-arabinoturanosylcytosine, 9-beta-D-arabinoturanosylguanine, 9-beta-D-arabinoturanosylguanine 5'-triphosphate, 1-beta-D-arabinoturanosylthymine, 5-azacytidine, 8-azaguanine, 3'-azido-3'-deoxythymidine, 6-beniylaminopurine, cytidine phosphoramidite, beta-cyanoethyl diisopropyl, 249802cytidine-5'-triphosphate, 2'-deoxyadenosine, 2'-deoxyadenosine 5'-triphosphate, 2'-deoxycytidine, 2'-deoxycytidine 5'-triphosphate, 2'-deoxyguanosine, 2'-deoxyguanosine 5'-triphosphate, 2',3'-dideoxyadenosine, 2',3'-dideoxyadenosine 5'-triphosphate, 2',3'-dideoxycytidine, 2',3'-dideoxycytidine 5'-triphosphate, 2',3'-dideoxyguanosine, 2',3'-dideoxyguanosine 5'-triphosphate, 2',3'-dideoxyinosine, 2',3' dideoxythymidine, 2',3'-dideoxythymidine 5'-triphosphate, 2',3'-dideoxyuridine, N6-dimethylallyladenine, 5-fluoro-2'-deoxyuridine, 5-fluorouracil, 5-fluorouridin, 5-fluorouridine 5'-monophosphate, formycine A 5'-triphosphate, formycine B, guanosine-3'-5'-cyclic monophosphate, guanosine-5'-diphosphate-3'-diphosphate, guanosine-5'-o-(2-thiotriphosphate), guanosine-5'-o-(3'-thiotriphosphate), guanosine 5'-triphosphate, 5'-guanylylimidodiphosphate, inosine, 5-iodo-2'-deoxyuridine, nicotinamide-adenine dinucleotides, nicotinamide-adenine dinucleotides, nicotinamide-adenine dinucleotide phosphate, oligodeoxythymidylic acid, (p(dT)10), oligodeoiythymidylic acid (p(dT)12-18), polyadenylic acid

(poly A), polyadenylic acid-oligodeoxythymidynic acid, polycytidylic acid, poly(deoxyadenyl-deoxiythymidylic acid, polydeoxyadenylic-acid-oligodeoxythymidynic acid, polydeoxythymidylic acid, polyinosine acid-polycytidylic acid, polyuridynic acid, ribonucleic acid, tetrahydro-uridine, thymidine, thymidine-3',5'-diphosphate, thymidine phosphoramidite, beta-cyanoethyl diisopropyl, 606102 thymidine 5'-triphosphate, thymine, thymine riboside, uracil, uridine, uridine-5'-diphosphoglucose, uridine 5'-triphosphate, xanthine, zeatine, transeatine riboside, etc. Further suitable polymers are: poly(DA) ss, poly(A) ss, poly(C) ss, poly(G) ss, poly(U) ss, poly(DA)-(DT) ds, complementary homopolymers, poly (D(A-T)) ds, copolymers, poly(DG)·(DC) ds, complementary homopolymers, poly (d(G-C)) ds copolymers, poly (d(L-C)) ds copolymers, poly(I)-poly(C) ds, etc. An oligopeptide or a polypeptide preferably contains 3-250, frequently 4-100, and very often 4-50 amino acids which are mutually coupled via amide-bonds. Suitable amino acids are usually of the alpha- and L-type; exceptions, however, such as in dermorphine are possible.

Peptides with a particularly high biological and/or therapeutic significance, and which can also be combined with transfersomes, are, for example, N-acetyl-Ala-Ala-Ala-, N-acetyl-Ala-Ala-Ala methyl ester, N-acetyl-Ala-Ala-Ala-Ala, N-acetyl-Asp-Glu, N-acetyl-Gly-Leu, Nalpha-Acetyl-Gly-Lys methyl ester acetate, acetyl-hirudine fragments, acetyl-5-hydroxy-Trp-5-hydroxy-Trp amide, des-acetyl-alpha-melanocyte stimulating hormone, N-Acetyl-Met-Asp-Arg-Val-Leu-Ser-Arg-Tyr, N-acetyl-Met-Leu-Phe, acetyl-muramyl-Ala-isoGln, N-acetyl-Phe-Tyr, N-acetyl-Phe-norLeu-Arg-Phe amide, N-acetyl-renine substrate tetradecapeptide, N-acetyl-transforming growth factor, adipokinetic hormone II, adjuvant peptide,

adrenal peptide E, adrenocorticotropic hormone (ACTH 1-39, Corticotropine A) and its fragments such as 1-4 (Ser-Tyr-Ser-Met), 1-10 (Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly), 1-17, 1-24 and 1-39, 11-24, 18-39, Ala-Ala, beta-Ala-Ala, Ala-Ala-Ala, Ala-Ala-Ala methyl ester, Ala-Ala-Ala-Ala, Ala-Ala-Ala-Ala-Ala, Ala-Ala-Ala-Ala-Ala-Ala, Ala-Ala-Phe, 7-amido-4-methylcoumarin, Ala-Ala-Phe p-nitroanilide, Ala-Ala-Val-Ala p-nitroanilide, Ala-Arg-Pro-Gly-Tyr-Leu-Ala-Phe-Pro-Arg-Met amide, beta-Ala-Arg-Ser-Ala-Pro-Thr-Pro-Met-Ser-Pro-Tyr, Ala-Asn, Ala-Asp, Ala-Glu, Ala-gamma-Gln-Lys-Ala-Ala, Ala-Gly, beta-Ala-Gly, Ala-Gly-Glu-Gly-Leu-Ser-Ser-Pro-Phe-Tyr-Ser-Leu-Ala-Ala-Pro-Gln-Arg-Phe amide, Ala-Gly-Gly, Ala-Gly-Ser-Glu, Ala-His, beta-Ala-His, Ala-isoGln-Lys-Ala-Ala, Ala-Ile, Ala-Leu, beta-Ala-Leu, Ala-Leu-Ala, Ala-Leu-Ala-Leu, Ala-Leu-Gly, Ala-Lys, beta-Ala-Lys, Ala-Met, N-beta-Ala-1-methyl-His, Ala-norVal, Ala-Phe, beta-Ala-Phe, Ala-Phe-Lys 7-amido-4-methylcoumarin, Ala-Pro, Ala-Pro-Gly, Ala-sarcosine, Ala-Ser, Ala-Ser-Thr-Thr-Thr-AsN-Tyr-Thr, Ala-Ser-Thr-Thr-Thr-Asn-Tyr-Thr amide, Ala-Thr, Ala-Trp, beta-Ala-Trp, Ala-Tyr, Ala-Val, beta-Ala-Val, beta-Ala-Trp-Met-Asp-Phe amide, alytesine, amanitine, amastatine, angiotensine I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu), II II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe), III and related peptides, angiotensine II antagonist, angiotensine II receptor binding protein, angiotensine converting enzyme and its inhibitor (e.g. entipaine, bestatine, chymostatine, E-64, elastatinal, etc.) anserine, antide, aprotinine, arginine, vasopressine-Ala-Gly, Arg-Ala, Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly, Arg-Asp, Arg-Glu, Arg-Gly, Arg-Gly-Asp, Arg-Gly-Asp-Ser, Arg-Gly-Asp-Ser-Pro-Ala-Ser-Ser-Lys-Pro, Arg-Gly-Glu-Ser, Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala, Arg-His-Phe, Arg-Ile, Arg-Leu, Arg-Lys, Arg-Lys-Asp-Val-Tyr, Arg-Phe, Arg-Phe-Asp-Ser, Arg-Pro-Pro-Gly-Phe-Ser-

Pro-Phe-Arg, Arg-Ser-Arg, Arg-Ser-Arg-His-Phe, Arg-Val, Asn-Pro-Asn-Ala-Asn-Pro-Asn-Ala, Asn-Pro-Asn-Ala-Asn-Pro-Asn-Ala-Asn-Pro-Asn-Ala, alpha-Asp-Ala, Asp-Ala-Glu-Asn-Leu-Ile-Asp-Ser-Phe-Gln-Glu-Ile-Val, Asp-Asp, alpha-Asp-Glu, alpha-Asp-Gly, beta-Asp-Gly, beta-Asp-His, Asp-Leu amide, beta-Asp-Leu, alpha-Asp-Lys, alpha-Asp-Phe amide, alpha-Asp-Phe, alpha-Asp-Phe methyl ester, beta-Asp-Phe methyl ester, alpha-Asp-Ser-Asp-Pro-Arg, Asp-Val, beta-Asp-Val, atrial natriuretic peptide, especially its fragments 1-32 and 5-28, atriopeptine I, II and III, auriculine A and B, beauvericine, beniotript, bestatine, N-benzylated peptides, big gastrine I, bombesine, (D-Phe12,Leu14) (Tyr4), (Lys3)-bombesine, (Tyr4)-bombesine, adrenal medulla docosapeptide and dodecapeptide, Bradykinine (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) and related peptides, Bradykinine potentiators, brain natriuretic peptide, buccaline, bursine, S-t-butyl-Cys, caeruleine, calcitonine, calcitonine gene related peptide I and II, calmoduline binding domain, N-carboxymethyl-Phe-Leu, N-((R,S)-2-carboxy-3-phenyl-propionyl)Leu, cardioactive peptides A and B, carnosine, beta-casomorphine, CD4, cerebelline, N-chloroacetyl-Gly-Gly, chemotactic peptides such as formylated substances, cholecystokinine fragments, e.g., cholecystokinine octapeptide, coherine etc.

Also worth mentioning are the collagen peptides, conicostatine, conicotropine releasing factor, conotoxin G1, M1, and GVIA, corticotropine-like intermediate lobe peptide, corticotropine releasing factor and related peptides, C-peptide, Tyr-C-peptide, cyclic calcitonine gene related peptides, cyclo(His-Phe-), cyclo(His-Pro-), cyclo(Leu-Gly-), cyclo(Pro-Gly-), Cys-Asp-Pro-Gly-Tyr-Ile-Ser-Arg amide, Cys-Gln-Asp-Ser-Glu-Thr-Arg-Thr-Phe-Tyr, DAGO, Delta-sleep inducing peptide, dermorphine,

(Ser(Ac)7)-dermorphine, diabetes associated peptide and its amide, N-alpha,N-epsilon-diacetyl-Lys-Ala-Ala, N-2,4-dinitrophenyl-Pro-Gln-Gly-Ile-la-Gly-Gln-Arg, diprotine A, dynorphines such as dynorphine A (Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-sn-Gln), fragments 1-6 (leucine encephaline-Arg), 1-8, 1-13 or E-64, dynorphine B, ebelactones (e.g. A and B) ecarine, elastatinal, eledoisine and related peptides, alpha-, beta- und gamma-endorphine, endothelins, endorphines (e.g. alpha (=beta-Lipotropine 61-76), (Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr), beta (=beta-Lipotropine 61-91) and other beta-lipotropine-fragments, encephaline and Leu-encephaline (Tyr-Gly-Gly-Phe-Leu) and related peptides, encephalinase inhibitors (e.g. epiamastatine, epibestatine, foroxymithine, leupeptine, pepstatine, Nle-Sta-Ala-Sta), eosinophilo-tactic tetrapeptide, epiamastatine, epibestatine, (Cys(Acm)20,31)-epidermal growth factor and its fragments or receptors, epidermal mitosis inhibiting pentapeptide, trans-epoxysuccinyl-Leu amido-(4-guanidino)butane, erythropoietine and fragment, S-ethylglutathione, fibrinogen related peptide, fibrinopeptide A and B, Tyr-fibrinopeptide A, (Glu1)-fibrinopeptide S, fibrinopeptide B-Tyr, fibroblast growth factor fragment 1-11, follicular gonadotropine releasing peptide, N-formylated peptides, foroxymithine, N-(3(2-furyl)acryloyl) peptide derivatives, galanine, GAP 1-13, gastric inhibitory polypeptide, gastrine related peptides and derivatives, gastrine releasing peptide, gastrointestinal peptides (e.g. Ala-Trp-Met-Asp-Phe-Amid, bombesine, caeruleine, cholecystokinine, gelanine, gastrine, glucagon, motiline, neuropeptide K, pancreatic polypeptide, pancreozymine, Phi-27, secretine, valosine, etc.), Gln-Ala-Thr-Val-Gly-Asp-Val-Asn-Thr-Asp-Arg-Pro-Gly-Leu-Leu-Asp-Leu-Lys, (des-His1, Glu9)-glucagon amide, glucagon (1-37),

glucagon-like peptide I, alpha-Glu-Ala, Glu-Ala-Glu, Glu-Ala-Glu-Asn, alpha-Glu-Glu, gamma-Glu-Glu, gamma-Glu-Gln, gamma-Glu-Gly, PGlu-Gly-Arg-Phe amide, alpha-Glu-Gly-Phe, gamma-Glu-His, gamma-Glu-Leu, alphaGlu-alpha-Lys, gamma-Glu-epsilon-Lys, N-gamma-Glu-Phe, PGlu-Ser-Leu-Arg-Trp amide, alpha-Glu-Trp, gamma-Glu-Trp, gamma-Glu-Tyr, alpha-Glu-Val, gamma-Glu-Val, PGlu-Val-Asn-Phe-Ser-Pro-Gly-Trp-Gly-Thr amide, A-Glu-Val-Phe, glutathiones and related peptides, glutathionesulfonic acid, Gly-Ala, Gly-beta-Ala, Gly-Ala-Ala, Gly-Ala-Ala-Ala-Ala, Gly-Ala-Tyr, Gly-alpha-aminobutyric acid, Gly-gamma-aminobutyric acid, Gly-Arg-Ala-Asp-Ser-Pro-Lys, Gly-Arg-Ala-Asp-Ser-Pro-OH, Gly-Arg-Gly-Asp-Ser, Gly-Arg-Gly-Asp-Asn-Pro-OH, Gly-Arg-Gly-Asp-Ser-OH, Gly-Arg-Gly-Asp-Ser-Pro-Lys, Gly-Arg-Gly-Asp-Ser-Pro-OH, Gly-Arg-Gly-Asp-Thr-Pro, Gly-Arg-Gly-Asp-Thr-Pro-OH, Gly-Arg p-nitroanilide, Gly-Arg-Gly-Asp, Gly-Arg-Gly-Asp-Ser, Gly-Asn, Gly-Asp, Gly-Asp-Asp-Asp-Asp-Lys, Gly-Glu, Gly-Gly and their derivatives such as methyl, ethyl or benzyl esters or amides, Gly-Gly-Ala, Gly-Gly-Arg, Gly-Gly-Gly, Gly-Gly-Gly-Gly, Gly-Gly-Gly-Gly-Gly, Gly-Gly-Gly-Gly-Gly-Gly, Gly-Gly-Ile, Gly-Gly-Leu, Gly-Gly-Phe, Gly-Gly-Phe-Leu, Gly-Gly-Phe-Leu amide, Gly-Gly-Phe-Met, Gly-Gly-Phe-Met amide, Gly-Gly-sarcosine, Gly-Gly-Tyr-Arg, Gly-Gly-Val, Gly-His, Gly-His-Arg-Pro, Gly-His-Gly, Gly-His-Lys, Gly-His-Lys-OH, Gly-Ile, Gly-Leu amide, Gly-Leu, Gly-Leu-Ala, Gly-Leu-Phe, Gly-Leu-Tyr, Gly-Lys, Gly-Met, Gly-norLeu, Gly-norVal, Gly-Phe amide, Gly-Phe, Gly-Phe-Ala, Gly-Phe-Arg, Gly-Phe-Leu, Gly-Phe-Phe, Gly-Pro, Gly-Pro-Ala, Gly-Pro-Arg, Gly-Pro-Arg-Pro, Gly-Pro-Arg-Pro-OH, Gly-Pro-Gly-Gly, Gly-Pro-hydroxy-Pro, Gly-sarcosine, Gly-Ser, Gly-Ser-Phe, Gly-Thr, Gly-Trp, Gly-Tyr amide, Gly-Tyr, Gly-Tyr-Ala, Gly-Val, Gly-Phe-Ser, granuliberine R, growth hormone releasing factor and its fragments, Hexa-Ala, Hexa-Gly, Hippuryl-Arg (Hip-Arg), Hippuryl-Gly-Gly (Hip-

Gly-Gly), Hippuryl-His-Leu (Hip-His-Leu), Hippuryl-Lys, Hippuryl-Phe, hirudine and its fragments, His-Ala, His-Gly, His-Leu, His-Leu-Gly-Leu-Ala-Arg, His-Lys, His-Phe, His-Ser, His-Tyr, HIV envelope protein (gp120), Hydra peptides, P-hydroxyhippuryl-His-Leu, hypercalcemia malignancy factor (1-40), insulin chains B and C, P-iodo-Phe, Ile-Asn, Ile-Pro-Ile, insulin-like growth factor I (especially fragment 1-70), insulin-like growth factor II (especially its fragment 33-40), interleukin-1B fragment 163-171, isotocine, kassinine (Asp-Val-Pro-Lys-Ser-Asp-AGly-n-Phe-Val-Gly-Leu-Met-NH_2) katacalcine (calcitonine precursor peptide), Tyr-katacalcine, kemptide, kentsine, kyotorphine, laminine nonapeptide, laminine pentapeptide, laminine pentapeptide amide, leucine encephaline and related peptides, leucopyro-kinine, Leu-Ala, Leu-beta-Ala, Leu-Arg, Leu-Asn, leucokinine I (Asp-Pro-Ala-Phe-Asn-Ser-Trp-Gly-NH_2) and II, Leucine-encephaline amide (Leu-encephaline amide) and related peptides, Leu-Gly, Leu-Gly-Gly, Leu-Gly-Phe, Leu-Leu amide, Leu-Leu, Leu-Leu-Leu amide, Leu-Leu-Leu, Leu-Leu-Phe amide, Leu-Leu-Tyr, Leu-Lys-Lys-Phe-Asn-Ala-Arg-Arg-Lys-Leu-Lys-Gly-Ala-Ile-Leu-Thr-Thr-Met-Leu-Ala, Leu-Met, Leu-Met-Tyr-Pro-Thr-Tyr-Leu-Lys, Leu-Phe, Leu-Pro, Leu-Pro-Pro-Ser-Arg, Leu-Ser, Leu-Ser-Phe, Leu-Trp, Leu-Tyr, Leu-Val, leucotriene, Leu-Leu methyl ester, leupeptin, Leu-Ser-p-nitro-Phe-Nle-Ala-Leu methyl ester, beta-lipotropin fragments, litorine, luteinizing hormone releasing hormone and related peptides, lymphocyte activating pentapeptide, Lys-Ala, Lys-Ala 7-amido-4-methylcoumarin, Lys-Asp, Lys-Cys-Thr-Cys-Cys-Ala, Lys-Glu-Glu-Ala-Glu, Lys-Gly, Lys-Leu, Lys-Lys, Lys-Met, Lys-Phe, Lys-Pro-Pro-Thr-Pro-Pro-Pro-Glu-Pro-Glu-Thr, Lys-Serum thymic factor, Lys-Trp-Lys, Lys-Tyr-Trp-Trp-Phe amide, Lys-Val, macrophage inhibitory peptide (Tuftsine

fragment 1-3, Thr-Lys-Pro), magainine I and II, mast cell degranulating peptide, mastoparane, alpha1-mating factor, Melanine-Concentrating Hormone, MCD peptide, alpha-, beta-, gamma-, and delta-melanocyte stimulating hormones and related peptides, melittine, mesotocine, Met-beta-Ala, Met-Asn-Tyr-Leu-Ala-Phe-Pro-Arg-Met amide, methionine encephaline and related peptides, Met-Ala, Met-Ala-Ser, Met-Asn, methionine-encephaline (Met-encephaline, Tyr-Gly-Gly-Phe-Met) and related peptides, methionine-encephaline amide (Met-Encephaline amide, Tyr-Gly-Gly-Phe-Met-NH_2) and related peptides, Met-Gln-Trp-Asn-Ser-Thr-Thr-Phe-His-Gln-Thr-Leu-Gln-Asp-Pro-Arg-Val-Arg-Gly-Leu-Tyr-Phe-Pro-Ala-Gly-Gly, Met-Glu, Met-Gly, Met-Leu, Met-Leu-Phe, Met-Lys, Met-Met, Metorphamide, Met-Phe, Met-Pro, Met-Ser, Met-Tyr-Phe amide, Met-Val, N-Methoxycarbonyl-Nle-Gly-Arg, P-nitroaniline, methoxy-succinyl-Ala-Ala-Pro-Val, methoxysuccinyl-Ala-Ala-Pro-Val 7-amido-4-methylcoumarin, Met-somatotropine, molluscan cardioexcitatory peptide, morphiceptine, (Val3)-morphiceptine, motiline, MSH-release inhibiting factor, myeline basic protein or its fragments, naphthyl-amide-derivatives of various peptides, beta-naphthyl-Ala-Cys-Tyr-Trp-Lys-Val-Cys-Thr amide, alpha-neoendorphine, beta-neoendorphine, alpha-neurokinin, neurokinin A, (substance K, neuromedin L) and B, neoendorphine (alpha: Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro, beta, etc.) neuromedin B, C, K, U8, U-25 etc., neurokinin A and B, neuropeptides K and Y, neurophysin I and II, neurotensine and related peptides, nitroanilide peptide derivatives, Nle-Sta-Ala-Sta, NorLeu-Arg-Phe amide, opioid peptides (e.g. adrenal peptide E, Ala-Gly-Glu-Gly-Leu-Ser-Ser-Pro-Phe-Trp-Ser-Leu-Ala-Ala-Pro-Gln-Arg-Phe-amides, casein fragments, casomorphine, N-CBZ-Pro-D-Leu, dermorphine, kyotorphine, morphiceptine (Tyr-Pro-Phe-Pro-NH2), meorph-amide (Tar-Gly-Gly-Phe-Met-Arg-Arg-Val, adrenorphine),

osteocalcin (esp. its fragment 7-19), oxytocine and related peptides, pancreastatine and its fragments, such as 33-49, pancreatic polypeptide, pancreozymin, parathyroid hormone or fragments thereof, especially 1-34 and 1-84, penta-Ala, penta-Gly, penta-Phe, pepstatin A, peptide YY, peptide T, phalloidin, Phe-Ala-Ala-p-nitro-Phe-Phe-Val-Leu 4-pyridylmethyl ester, Phe-Leu-Phe-Gln-Pro-Gln-Arg-Phe amide, Phe-Ala, Phe-Gly, Phe-Gly-Gly, Phe-Gly-Gly-Phe, Phe-Gly-Phe-Gly, Phe-Leu amide, Phe-Leu, Phe-Leu-Arg-Phe amide, Phe-Leu-Glu-Glu-Ile, Phe-Leu-Glu-Glu-Leu, Phe-Leu-Glu-Glu-Val, Phe-Met, Phe-Met-Arg-Phe amide, Phe-Phe, Phe-Phe-Phe, Phe-Phe-Phe-Phe, Phe-Phe-Phe-Phe-Phe, Phe-Pro, Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Arg, Phe-Tyr, Phe-Val, PHI-27, PHM-27, phosphoramidone, physalaemine (pGlu-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu-Met-NH2), preproencephaline fragment 128-140, pressinoic acid and related peptides, Pro-Asn, proctoline (Arg-Tyr-Leu-Pro-Thr), proencephaline, Pro-His-Pro-Phe-His-Phe-Phe-Val-Tyr-Lys, Pro-Ala, Pro-Arg 4-methoxy-beta-naphthylamide, Pro-Asp, proglumide, Pro-Gly, Pro-Gly-Gly, Pro-hydroxy-Pro, Pro-Ile, Pro-Leu, Pro-Leu-Gly amide, Pro-Met, Pro-Phe amide, Pro-Phe, Pro-Phe-Arg 7-amido-4-methylcoumarin, Pro-Phe-Gly-Lys, Pro-Trp, Pro-Tyr, Pro-Val, cyclic AMP dependent protein kinase and its inhibitors, PyroGlu-Ala-Glu, PyroGlu-Ala, PyroGlu-Ala-Glu, PyroGlu-Asn-Gly, PyroGlu-Gly-Arg p-nitroanilide, PyroGlu-His-Gly amide, PyroGlu-His-Gly, PyroGlu-His-Pro amide, PyroGlu-His-Pro, PyroGlu-Lys-Trp-Ala-Pro, ranatensine, renine substrate tetradecapeptide, N-(alpha-rhamnopyranosyloxy-hydroxyphosphinyl) Leu-Trp, sarcosyl-Pro-Arg p-nitroanilide, sauvagine, sleep-inducing peptide (Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu), secretine and related peptides, Ser-Ile-Gly-Ser-Leu-Ala-Lys, Ser-Ser-Ser, serum thymic factor, Ser-Ala, Ser-beta-Ala, Ser-Asn, Ser-Asp, Ser-Asp-Gly-Arg-Gly, Ser-Glu, Ser-Gln, Ser-Gly,

Ser-His, Ser-Leu, Ser-Met, Ser-Phe, Ser-Ser-Ser, Ser-Tyr, sleep inducing peptide, somastotine and related peptides (e.g. cyclo(p-Trp-Lys-Trh-Phe-Pro-Phe), steroido-genesis activator polypeptide, substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH2) and related peptides, N-succinyl-derivatives of various peptides, syndyphalin-20 (Tyr-D-Met(O)-Gly-Phe-ol), tentoxin, tetra-Ala, tetra-Gly, thiostrepton, DL-thiorphane (encephalinase inhibitor), Thr-beta-Ala, Thr-Asp, Thr-Leu, Thr-Lys-Pro-Arg, Thr-Ser, Thr-Ser-Lys, Thr-Tyr-Ser, Thr-Val-Leu, thymopoietin fragments, thymosin alpha1 and its fragments, thymus circulating factor, thyrocalicitonin, thyrotropin releasing hormone, tocinoic acid, tosylated peptides, transforming growth factors, Tri-Ala, Tri-Ala methyl ester, Trp-Ala, Trp-Ala-Trp-Phe amide, Trp-Glu, Trp-Gly, Trp-Gly-Gly, Trp-His-Trp-Leu-Gln-Leu, Trp-His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Met-Tyr, Trp-His-Trp-Leu-Ser-Phe-Ser-Lys-Gly-Glu-Pro-Met-Tyr, Trp-Leu, Trp-Met-Asp-Phe amide, Trp-norLeu-Arg-Phe amide, Trp-Phe, Trp-Trp, Trp-Tyr, Tuftsin (Thr-Lys-Pro-Arg) and its fragments, Tyr-Ala, Tyr-Ala-Gly, Tyr-Ala-Gly-Ala-Val-Val-Asn-Asp-Leu, Tyr-Ala-Gly-N-methyl-Phe 2-hydroxyethylamide, Tyr-Ala-Phe-Met amide, Tyr-Arg, Tyr-atriopeptin II, Tyr-Glu, Tyr-Gly, Tyr-Gly-Ala-Val-Val-Asn-Asp-Leu, Tyr-Gly-Gly, Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Arg, Tyr-Gly-Gly-Phe-Met-Arg-Arg-Val amide, Tyr-Gly-Trp-Phe-Phe amide, Tyr-Leu, Tyr-Phe, Tyr-Phe-Met-Arg-Phe amide, Tyr-Phe-Phe amide, Tyr-Pro-Leu-Gly amide, Tyr-Pro-Phe-Pro amide, Tyr-Pro-Val-Pro amide, Tyr-Thr-Gly-Leu-Phe-Thr, Tyr-Tyr-Phe amide, Tyr-Trp-Ala-Trp-Phe amide, Tyr-Trp-Ala-Trp-Phe methylamide, Tyr-Tyr-Leu, Tyr-Tyr-Phe, Tyr-Tyr-Tyr, Tyr-Tyr-Tyr methyl ester, Tyr-Tyr-Tyr-Tyr-Tyr-Tyr, Tyr-Val amide, Tyr-Val, Tyr-Val-Gly, Urodilatin, Urotensin II, Valosin, Val-Ala, Val-Ala p-nitroanilide, Val-Ala-Ala-Phe, Val-Asp, Val-Glu, Val-Gln, Val-Glu-Glu-Ala-Glu, Val-Glu-Ser-Ser-Lys,

Val-Gly, Val-Gly-Asp-Gln, Val-Gly-Gly, Val-Gly-Ser-Glu, Val-Gly-Val-Ala-Pro-Gly, Val-His-Leu-Thr-Pro, Val-His-Leu-Thr-Pro-Val-Glu-Lys, Val-Leu, Val-Lys, Val-Met, Val-Phe, Val-Pro, Val-Pro-Asp-Pro-Arg, Val-Pro-Leu, Val-Ser, Val-Thr, Val-Trp, Val-Tyr, Val-Tyr-Val, Val-Val, vasoactive intestinal peptides and related peptides, vasopressin related peptides, vasotocin and related peptides, xenopsin, etc.

Extended polypeptides are normally called proteins, independent of their detailed conformation. In this description, this term denotes, by and large, an enzyme or a coenzyme, an adhesion- or a recognition molecule, such as a CAMP or an OMP or a lectin, a histocompatibility complex, such as MHC-I or MHC-II, or an immunoglobuline (antibody) - or any (bio)chemical or (molecular)genetic modification thereof. Particularly useful for the applications according to this invention are the (bio)chemical modifications in which individual proteins are substituted with apolar residues, such as an alkyl, acyl, alkenoyl, etc. chains; but this is not a stringent limitation.

An enzyme is a catalytically active protein. Enzymes are normally grouped according to their basic functions. The most important enzymes for this invention are (E.C. numbers are given in brackets):

Oxidoreductases, such as: alcohol dehydrogenase (1.1.1.1), alcohol dehydrogenase (NADP dependent) (1.1.1.2), glycerol dehydrogenase (1.1.1.6), glycerophosphate dehydrogenase (1.1.1.8), xylulose reductase (1.1.1.10), polyol dehydrogenase (1.1.1.14), sorbitol dehydrogenase (1.1.1.14), myo-inositol dehydrogenase (1.1.1.18), uridine 5'-diphosphoglucose dehydrogenase

(1.1.1.22), glyoxalate reductase (1.1.1.26), lactate dehydrogenase (1.1.1.27), lactate dehydrogenase (1.1.1.28), glycerate dehydrogenase (1.1.1.29), beta-hydroxybutyrate dehydrogenase (1.1.1.30), beta-hydroxyacyl CoA dehydrogenase (1.1.1.35), malate dehydrogenase (1.1.1.37), malate enzyme (1.1.1.40), isocitric dehydrogenase (1.1.1.42), 6-phosphogluconate dehydrogenase (1.1.1.44), glucose dehydrogenase (1.1.1.47), beta-galactose dehydrogenase (1.1.1.48), glucose-6-phosphate dehydrogenase (1.1.1.49), 3alpha-hydroxysteroid dehydrogenase (1.1.1.50), 3beta-hydroxysteroid dehydrogenase (1.1.1.51), 3alpha,2beta-hydroxysteroid dehydrogenase (1.1.1.53), 3-phosphoglycerate dehydrogenase (1.1.1.95), fucose dehydrogenase (1.1.1.122), lactate dehydrogenase (cytochrome) (1.1.2.3), glucose oxidase (1.1.3.4), cholesterol oxidase (1.1.3.6), galactose oxidase (1.1.3.9), alcohol oxidase (1.1.3.13), glycolate oxidase (1.1.3.15), choline oxidase (1.1.3.17), glycerol-3-phosphate oxidase (1.1.3.21), xanthine oxidase (1.1.3.22), alcohol dehydrogenase (1.1.99.8), fructose dehydrogenase (1.1.99.11), formaldehyde dehydrogenase (1.2.1.1), formate dehydrogenase (1.2.1.2), aldehyde dehydrogenase (1.2.1.5), glyceraldehyde-3-phosphate dehydrogenase (1.2.1.12), gabase (1.2.1.16), pyruvate oxidase (1.2.3.3), oxalate oxidase (1.2.3.4), dihydroorotate dehydrogenase (1.3.3.1), lipoxidase (1.3.11.12), alanine dehydrogenase (1.4.1.1), glutamic dehydrogenase (1.4.1.3), glutamate dehydrogenase (NADP) (1.4.1.4), L-amino acid oxidase (1.4.3.2), D-amino acid oxidase (1.4.3.3), monoaminoxidase (1.4.3.4), diaminoxidase (1.4.3.6), dihydrofolate reductase (1.5.1.3), 5,10-methylenetetrahydrofolat dehydrogenase (1.5.1.5), saccharopine dehydrogenase NAD+ (1.5.1.7), octopine dehydrogenase (1.5.1.11), sarcosine oxidase (1.5.3.1),

sarcosine dehydrogenase (1.5.99.1), glutathione reductase (1.6.4.2), ferridoxin-NADP+ reductase (1.6.7.1), NADPH-FMN oxidoreductase (1.6.99.1), cytochrome c reductase (1.6.99.3), NADH-fmn oxidoreductase (1.6.99.3), dihydropteridin reductase (1.6.99.7), uricase (1.7.3.3), diaphorase (1.8.1.4), lipoamide dehydrogenase (1.8.1.4), cytochrome oxidase (1.9.3.1), nitrate reductase (1.9.6.1), phenolase (1.10.3.1), ceruloplasmine (1.10.3.2), ascorbate oxidase (1.10.3.3), NADH peroxidase (1.11.1.1), catalase (1.11.1.6), lactoperoxidase (1.11.1.7), myeloperoxidase (1.11.1.7), peroxidase (1.11.1.7), glutathione peroxidase (1.11.1.9), chloroperoxidase (1.11.1.10), lipoxidase (1.13.1.12), protocatechuate 3,4-dioxygenase (1.13.11.3), luciferase (glow-worm) (1.13.12.7), salicylate hydroxylase (1.14.13.7), p-hydroxybenzoate hydroxylase (1.14.13.2), luciferase (bacterial) (1.14.14.3), phenylalanine hydroxylase (1.14.16.1), dopamine-beta-hydroxylase (1.14.17.1), tyrosinase (1.14.18.1), superoxide dismutase (1.15.1.1), ferredoxine-NADP reductase (1.18.1.2), etc.. Transferases, such as: catecholic o-methyltransferase (2.1.1.6), phenylethanol-amine N-methyl-transferase (2.1.1.28), aspartate transcarbamylase (2.1.3.2), ornithine carbamyltransferase (2.1.3.3), transketolase (2.2.1.1), transaldolase (2.2.1.2), choline acetyltransferase (2.3.1.6), carnitine acetyltransferase (2.3.1.7), phosphotransacetylase (2.3.1.8), chloroamphenicol acetyltranferase (2.3.1.28), kanamycine 6'-acetyltransferase (2.3.1.55), gentamicine acetyltransferase (2.3.1.60), transglutaminase (2.3.2.13), gamma-glutamyl transpeptidase (2.3.2.2), phosphorylase A (2.4.1.1), phosphorylase B (2.4.1.1), dextransucrase (2.4.1.5), sucrose phosphornase (2.4.1.7), glycogen synthase (2.4.1.11), uridine 6'-diphospho-glucuronyltransferase (2.4.1.17), galactosyl trans-

ferase (2.4.1.22), nucleoside phosphorylase (2.4.2.1), orotidine-5'-monophosphate pyrophosphorylase (2.4.2.10), glutathione s-transferase (2.5.1.18), glutamine-oxalate transaminase (2.6.1.1), glutamic-pyruvate transaminase (2.6.1.2), gabase (2.6.1.19), hexokinase (2.7.1.1), galactokinase (2.7.1.6), fructose-9-phosphate kinase (2.7.1.11), gluconate kinase (2.7.1.12), phosphoribulokinase (2.7.1.19), NAD kinase (nicotinamide adenine dinucleotide kinase) (2.7.1.23), glycerokinase (2.7.1.30), choline kinase (2.7.1.32), protein kinase (3':5'-cyclic-AMP dependent) (2.7.1.37), phosphorylase kinase (2.7.1.38), pyruvate kinase (2.7.1.40), fructose-9-phosphate kinase (pyrophosphate dependent) (2.7.1.50), acetate kinase (2.7.2.1), carbamate kinase (2.7.2.2), 3-phosphoglyceric phosphokinase (2.7.2.3), creatine phosphokinase (2.7.3.2), etc.

Transpeptidases, such as: esterase (3.1.1.1), lipase (3.1.1.3), phospholipase A (3.1.1.4), acetylesterase (3.1.1.6), cholinesterase, acetyl (3.1.1.7), choline-esterase, butyryl (3.1.1.8), pectinesterase (3.1.1.11), cholesterol esterase (3.1.1.13), glyoxalase ii (3.1.2.6), phosphatase, alkaline (3.1.3.1), phosphatase acid (3.1.3.2), 5'-nucleotidase (3.1.3.5), 3'-nucleotidase (3.1.3.6), glucose-6-phosphatase (3.1.3.9), fructose-1,6-diphosphatase (3.1.3.11), phytase (3.1.3.26), phosphodiesterase i (3.1.4.1), glycerophosphorylcholine (3.1.4.2), phospholipase C (3.1.4.3), phospholipase D (3.1.4.4), deoxyribonuclease I (3.1.4.5), deoxyribonuclease II (3.1.4.6), ribonuclease N1 (3.1.4.8), sphingomyelinase (3.1.4.12), phosphodiesterase 3':5'-cyclic (3.1.4.17), phosphodiesterase II (3.1.4.18), endonuclease (3.1.4.21), ribonuclease A (3.1.4.22), ribonuclease B (3.1.4.22), 3'-phosphodiesterase 2':3'-cyclic nucleotide (3.1.4.37), sulfatase (3.1.6.1), chondro-4-sulfatase (3.1.6.9),

chondro-6-sulfatase (3.1.6.10), ribonuclease T2 (3.1.27.1), ribonuclease T1 (3.1.27.3), ribonuclease u2 (3.1.27.4), nuclease (3.1.30.1), nuclease, (from micrococces) (3.1.31.1), alpha-amylase (3.2.1.1), beta-amylase (3.2.1.2), amyloglucosidase (3.2.1.3), cellulase (3.2.1.4), laminarinase (3.2.1.6), dextranase (3.2.1.11), chitinase (3.2.1.14), pectinase (3.2.1.15), lysozyme (3.2.1.17), neuraminidase (3.2.1.18), alpha-glucosidase, maltase (3.2.1.20), beta-glucosidase (3.2.1.21), alpha-galactosidase (3.2.1.22), beta-galactosidase (3.2.1.23), alpha-mannosidase (3.2.1.24), beta-mannosidase (3.2.1.25), invertase (3.2.1.26), trehalase (3.2.1.28), beta-N-acetylglucosaminidase (3.2.1.30), beta-glucuronidase (3.2.1.31), hyaluronidase (3.2.1.35), beta-xylosidase (3.2.1.37), hesperidinase (3.2.1.40), pullulanase (3.2.1.41), alpha-fucosidase (3.2.1.51), mycodextranase (3.2.1.61), agarase (3.2.1.81), endoglycosidase F (3.2.1.96), endo-alpha-N-acetylgalactosaminidase (3.2.1.97), NADase (nicotinamide adenine glycopeptidase) F (3.2.2.5), dinucleotidase (3.2.2.18), thiogluc (3.2.3.1), s-adenosylhomocystein-hydrolase (3.3.1.1), leucin-aminopeptidase, (from cytosol) (3.4.11.1), leucin-aminopeptidase, microsomale (3.4.11.2), pyroglutamate-aminopeptidase (3.4.11.8), carboxypeptidase a (3.4.12.2), carboxypeptidase B (3.4.12.3), prolidase (3.4.13.9), cathepsin C (3.4.14.1), carboxypeptidase W (3.4.16.1), carboxypeptidase A (3.4.17.1), carboxypeptidase B (3.4.17.2), alpha-chymotrypsin (3.4.21.1), beta-chymotrypsin (3.4.21.1), gamma-chymotrypsin (3.4.21.1), delta-chymotrypsin (3.4.21.1), trypsin (3.4.21.4), thrombin (3.4.21.5), plasmin (3.4.21.7), kallikrein (3.4.21.8), enterokinase (3.4.21.9), elastase from pancreas (3.4.21.11), protease (subtilisin) (3.4.21.14), urokinase (3.4.21.31), elastase from leucocytes (3.4.21.37), cathepsin B, (3.4.22.1), papain (3.4.22:2),

ficin (3.4.22.3), bromo-elain (3.4.22.4), chymopapain (3.4.22.6), clostripain (3.4.22.8), proteinase A (3.4.22.9), pepsine (3.4.23.1), renine (3.4.23.4), cathepsin D (3.4.23.5), protease (aspergillopeptidase) (3.4.23.6), collagenase (3.4.24.3), collagenase (3.4.24.8), pinguinain (3.4.99.18), renine (3.4.99.19), urokinase (3.4.99.26), asparaginase (3.5.1.1), glutaminase (3.5.1.2), urease (3.5.1.5), acylase i (3.5.1.14), cholylglycine hydrolase (3.5.1.24), urease(ATP-hydrolyzing) (3.5.1.45), penicillinase (3.5.2.6), cephalosporinase (3.5.2.8), creatininase (3.5.2.10), arginase (3.5.3.1), creatinase (3.5.3.3), guanase (3.5.4.3), adenosine-deaminase (3.5.4.4), 5'-adenylate acid-deaminase (3.5.4.6), creatinine deiminase (3.5.4.21), anorganic pyrophosphatase (3.6.1.1), adenosine 5'-triphosphatase (3.6.1.3), apyrase (3.6.1.5), pyrophosphatase, nucleotide (3.6.1.9), etc.

Lyases, such as: pyruvate-decarboxylase (4.1.1.1), oxalate decarboxylase (4.1.1.2), oxalacetate decarboxylase (4.1.1.3), glutamic decarboxylase (4.1.1.15), ornithine decarboxylase (4.1.1.17), lysine decarboxylase (4.1.1.18), arginin decarboxylase (4.1.1.19), histidine decarboxylase (4.1.1.22), orotidine 5'-monophosphate decarboxylase (4.1.1.23), tyrosine decarboxylase (4.1.1.25), phospho(enol) pyruvate carboxylase (4.1.1.31), ribulose-1,5-diphosphate carboxylase (4.1.1.39), phenylalanine decarboxylase (4.1.1.53), hydroxymandelonitrilelyase (4.1.2.11), aldolase (4.1.2.13), N-acetylneuramine acid aldolase (4.1.3.3), etc. citrate lyase (4.1.3.6), citrate synthase (4.1.3.7), tryptophanase (4.1.99.1), isozymes of carbonic anhydrase (4.2.1.1), fumarase (4.2.1.2), aconitase (4.2.1.3), enolase (4.2.1.11), crotonase (4.2.1.17), delta-amino-levulinate dehydratase (4.2.1.24), chondroitinase ABC

(4.2.2.4), chondroitinase AC (4.2.2.5), pectolyase (4.2.2.10), aspartase (4.3.1.1), histidase (4.3.1.3), phenylalanine ammonia-lyase (4.3.1.5), argininosuccinate lyase (4.3.2.1), adenylosuccinate lyase (4.3.2.2), glyoxalase II (4.4.1.5), isomerases, such as: ribulose-5'-phosphate 3-epimerase (5.1.3.1), uridine 5'-diphosphogalactose 4-epimerase (5.1.3.2), mutarotase (5.1.3.3), triosephosphate isomerase (5.3.1.1), phosphoriboisomerase (5.3.1.6), phosphomannose isomerase (5.3.1.8), phosphoglucose isomerase (5.3.1.9), tautomerase (5.3.2.1), phosphoglucomutase (5.4.2.2), ligases, e.g.: aminoacyl-tRNA synthetase (6.1.1), s-acetyl coenzyme A synthetase (6.2.1.1), succinic thiokinase (6.2.1.4), glutamine synthetase (6.3.1.2), pyruvate carboxylase (6.4.1.1), etc.

The following are, amongst others, referred to as proteases: aminopeptidase M, amino acid-arylamidase, bromo-elaine, carboxypeptidase A, carboxypeptidase B, carboxypeptidase P, carboxypeptidase Y, cathepsine C, chymotrypsine, collagenases, collagenase/dispase, dispase, elastase, endoproteinase Arg-c, endoproteinase Asp-n sequencing grade, encloproteinase Glu-c (proteinase V8), endoproteinase Glu-c sequencing grade, endoproteinase Lys-c, endoproteinase Lys-c sequencing grade, endoproteinases, factor Xa, ficine, kallikrein, leucine-aminopeptidase, papaine, pepsine, plasmin, pronase, proteinase K, proteinase V8 (endoproteinase Glu-c), pyroglutamate-aminopeptidase, pyroglutamate-aminopeptidase, restriction protease factor Xa, subtilisine, thermolysine, thrombine, trypsine, etc.

A coenzyme according to this invention is any substance which supports enzyme activity. Amongst the biologically important coenzymes are, for example, acetyl-coenzyme A,

acetylpyridine-adenine-dinucleotide, coenzyme A, flavine-adenine-dinucleotide, flavine-mononucleotide, NAD, NADH, NADP, NADPh, nicotinamide-mononucleotide, s-palmitoyl-coenzyme A, pyridoxal-5'-phosphoric acid, etc.

Another class of proteins, which are important in the context of this invention, are lectins. Plants, and sometimes also animal, tissues are suitable sources of lectins; particularly convenient sources are Abrus pregatorius, Agarigus bisporus, Agrostemma githago, Anguilla anguilla, Arachis hypogaea, Artogarpus integrifolia, Bandeiraea simplicifolia BS-I und BS-II, (Griffonia simplicifolia), Banhlula purpurea, Caragana arborescens, Cicer arietinum, Canavalia ensiformis (jack bean), Caragana arborescens (Siberian pea tree), Codium fragile (green algae), Concanavalin A (Con A), Cytisus scoparius, Datura stramonium, Dolichos biflorus, Erythrina corallodendron, Euonymus europaeus, Gelonium multiflorum, Glycine max (soy), Griffonia simplicifolia, Helix aspersa (garden snail), Helix pomatia (escargot), Laburnum alpinum, Lathyrus odoratus, Lens culinaris (lentil), Limulus polyphemus, Lycopersicon esculentum (tomato), Lotus tetragonolobus, Luffa aegyptiaca, Maclura pomifera (Osaga orange), Momordica charantia (bitter pear melon), Naja mocambique (Mozambiquan cobra), Naja Naja kaouthia, Mycoplasma gallisepticum, Perseau americana (avocado), Phaseolus coccineus (beans), Phaseolus limensis, Phaseolus lunatus, Phaseolus vulgaris, Phytolacga americana, Pseudomonas aeruginosa PA-I, Pisum sativum (pea), Ptilota plumosa (red algae), Psophocarpus tetragonolobus (winged bean), Ricinus communis (castor bean), Robinia pseudoacacia (false acacia, black locust), Sambucus nigra (clematis), Saponaria officinalis, Solanum tuberosum (potato), Sophora japonica, Tetragonolobus purpureas (winged or asparagus pea), (Lotus tetragono-

lobus), Tritigum vulgaris (wheat germ), Ulex europaeus, Vicia faba, Vicia sativa, Vicia villosa, Vigna radiata, Viscum album (mistle), Wisteria floribunda, etc.

Further interesting proteins are, e.g. the activator of tissue-plasminogen, insulin, kallikrein, keratin, kininogene, lactoterrin, laminarin, laminin, alpha2-macroglobuline, alpha1-microglobuline, F2-microglobuline, high density lipoproteins, basic myeline-protein, myoglobine, neurofilaments I, II, and III, neurotensine, oxytocine, pancreatic oncofoetal antigen, parvalbumin, plasminogen, platelet factor 4, pokeweed antiviral protein, porphobilinogen, prealbumin, prostate specific antigens, protamine sulfate, protein C, protein C activator, protein S, prothrombin, retinol binding protein, S-100 protein, pregnancy protein-1, serum amyloid A, serum amyloid P component, tenascine, testosterone-estradiol binding globuline, thioredoxine, thrombine, thrombocytine, beta-thromboglobuline, thromboplastine, microsomal antigen from thyroidea, thyroidea stimulating hormone, thyroxine binding globuline, transcortine, transferrine, ubiquitine, vimentine, vinculine, vitronectine, etc.

Some typical examples of human and animal hormones which can be used as agents according to the invention are, for example, acetylcholine, adrenaline, adrenocorticotropic hormone, angiotensine, antidiuretic hormone, cholecystokinine, chorionic gonadotropine, corticotropine A, danazol, diethylstilbestrol, diethylstilbestrol glucuronide, 13,14-dihydro-15-keto-prostaglandins, 1-(3',4'-dihydroxyphenyl)-2-aminoethanol, 5,6-dihydroxy-tryptamine, epinephrine, follicle stimulating hormone, gastrin, gonadotropin, ß-hypophamine, insulin, juvenile hormone, 6-ketoprostaglandins, 15-ketoprostaglandins,

LTH, luteinizing hormone releasing hormone, luteotropic hormone, α-melanocyte stimulating hormone, gamma-melanocyte stimulating hormone, 5-melanocyte stimulating hormone, noradrenaline, norepinephrine, oxytocine, parathyroid hormone, parathyroid substances, prolactine, prostaglandins, secretine, somatostatine, somatotropine (STH), thymosine alpha 1, thyrocalcitonine, thyroglobuline, thyroid stimulating hormone, thyrotropic hormone, thyrotropine releasing hormone, 3,3',5-triiodothyroacetic acid, 3,3',5'-triiodothyronine, TSH, vasopressine, etc.

Oestrogens are mostly steroid hormones with 18 carbon atoms and one unsaturated (aromatic) ring. Amongst the most important oestrogens are, for example, chlorotrianisene, diencestrole, diethylstilboestrole, diethylstilboestrol-dipropionate, diethylstilboestroldisulfate, dimestrole, estradiole, estradiolbenzoate, estradiolundecylate, estriolsuccinate, estrone, ethinglestradiole, nexoestrole, nestranole, oestradiolvalerate, oestriole and quinestrole.

Gestagenes are typically synthetic hormones, mainly with progesterone-like characteristics; the most important agents belonging to this class are allylestrenole, chloromadinonacetate, dimethisterone, ethisterone, hydroxyprogesteron-caproate, lynestrenole, medrogestone, medroxyprogesteron-acetate, megestrolacetate, methyloestrenolone, norethisterone, norethisterone-acetate, and norgestrel.

Agents can also be parts of a biological extract. As sources of biologically and/or pharmacologically active extracts, the following are worth-mentioning: for example, Acetobacter pasteurianum, Acokanthera ouabaïo -

cathel, Aesculus hippocastanum, Ammi visnaga Lam., Ampi Huasca, Apocynum Cannabium, Arthrobotrys superba var. oligospora (ATCC 11572), Atropa belladonna, Bacillus Lentus, Bacillus polymyxa, Bacillus sphaericus, Castilloa elastica cerv., Chondrodendron tomentosum (Ampi Huasca), Convallaria majalis, Coronilla-enzymes, Corynebacterium hoagii (ATCC 7005), Corynebacterium simplex, Curvularia lunata (Wakker) Boadijn, Cylindrocarpon radicola (ATCC 11011), Cynara scolymus, Datura Metel, didymella, digilanidase, digitalis Lanata, digitalis purpurea, Duboisia, Flavobacterium dehydrogenans, Fusarium exquiseti saccardo, Hyoscyamus niger, Jaborandi-leaves (P. microphyilus Stapf), Micromonosporapurpurea u. echinospora, Paecilomyces varioti Bainier var. antibioticus, Penicillium chrysogenum Thom, Penicillium notatum Westling, Penicillium patulum, Rauwolfia serpentina Benth., Rhizopus arrhizus Fischer (ATCC-11145), Saccharomyces cerevisiae, Schizomycetes ATCC-7063, Scilla maritima L., Scillarenase, Septomyxa affinis (ATCC 6737), Silybum marianum Gaertn., Streptomyces ambofaciens, Strophantusgratus, Strophantus Kombe, Thevetia peruviana, Vinca minor L., Vinca rosea, etc.

Unless stated otherwise, all substances, surfactants, lipids, agents or additives with one or several chiral carbon atoms can be used either as a racemic mixture or in the form of optically pure enantiomers.

WORKING PRINCIPLE

The transport of agents through permeation barriers can be mediated by such carriers which fulfill the following basic criteria:

- carriers should experience or create a gradient which drives them into or through a barrier, e.g. from the body surface into or through the skin, or from the surface of a leaf into the depth of a leaf, or from one side of a barrier to the other;

- the resistance to permeation which is felt by the carriers in the barrier should be as small as possible in comparison to the driving force;

- carriers should be capable of permeating in and/or through a barrier without thereby losing their associated agents in an uncontrollable manner.

Carriers, moreover, should preferably provide control of the distribution of agents, as well as over the effectiveness and temporal development of the agents action. They should be capable of bringing materials into the depth of and across a barrier, if so desired, and/or should be capable of catalyzing such a transport. Last but not least, such carriers should affect the range and depth of action as well as the type of cells, tissue parts, organs and or system parts which can be reached or treated, under suitable conditions at least.

In the first respect, chemical gradients are especially convenient for biological applications. Particularly suitable are the physico-chemical gradients, such as the pressure of (de)hydration pressure (humidity gradient) or a difference in concentration between the sites of application and action; however, electrical or magnetic fields as well as thermal gradients are also interesting in this respect. In technological applications, an externally applied pressure or existing hydrostatic pressure difference are also of importance.

In order to fulfill the second condition, carriers must be sufficiently 'fluid' at the microscopic scale; this enables them to easily cross the constrictions in the permeability barrier.

Permeation resistance is a decreasing function of the decreasing carrier size. But also the carrier driving force frequently depends on the size of the permeating particle, droplet or vesicle; when the driving pressure is size-independent, the corresponding force also typically decreases with decreasing carrier size. This causes the transfer effectiveness to be a complex function of the carrier size, often showing a maximum depending on the chosen carrier and/or agent composition.

In the case of molecular aggregates the permeation resistance is largely determined by the mechanical elasticity and deformability of the carrier, the viscosity of the total preparation being also important, however. The former must be sufficiently high, the latter low enough.

Size and, even better, deformability can serve as a criterion for the optimization of the supramolecular carriers according to this invention. As an indication of deformability, the capacity of individual carriers to form protrusions can be studied, as a function of all relevant system parameters. (In practical terms, it is often sufficient to investigate only such variables which come into question for a controllable application. The examples given in this application, therefore, only pertain to varying the concentrations of the edge active components and the absolute carrier concentration which affect the forced diminishment of the lipid vesicle or of vesicle permeation.) This is true e.g. for transcutaneous and transcuticular transport as well as for the transport of agents through the lung alveoly, into the hair, into gels, and

the like.

With regard to the third requirement, the choice of the carriers, agents and additives, as well as the applied carrier dose or concentration all play some role. Low dose, in the majority of cases, gives rise to a predominantly surface treatment: poorly water-soluble substances in such case remain confined largely to the apolar region of a permeability barrier (such as in the epidermal membranes); agents which are highly soluble and can diffuse easily from the carriers can attain a distribution which is different from that of the carrier particles; for such substances, the permeability of a transfersomal membrane is also important. Edge active substances with a tendency to leave carriers and move into a barrier give rise to a locally variable carrier composition, etc. These interdependencies should be thought of and considered prior to each individual application. In the search for a set of conditions under which a simple carrier vesicle becomes a transfersome, the following rules of thumb can be used:

- At first, the conditions are determined under which the carrier vesicles are solubilized by the edge active substances. At this critical point the 'vesicles' are maximally deformable owing to the fact that they are permanently formed and deformed. At the same time, however, they are also unstable and incapable of holding and transferring water soluble substances.

- Next, the carrier composition or concentration is adapted by reducing the edge activity in the system to an extent which ensures the vesicle stability as well vesicle deformability to be sufficiently high; this also ensures the permeation capacity of such carriers to be satisfactory. The term stability in this application implies,

on the one hand, a mechanical tendency of the carrier components to "stay together"; on the other hand, that the carrier composition during the transport, and in particular during the permeation process, does not change at all or not much. The position of the corresponding optimum which one is looking for hereby depends on many boundary conditions. The type of agent molecules also plays an important role in this. The smaller and the more hydrophilic the agent to be transported, the further the carrier system must be spaced from the solubilization point; the desired shelf life of carriers is also important: upon approaching the solubilization point, the tendency of transfersomes to form larger particles may increase and the carrier's storage capacity simultaneously decrease.

- Ultimately, the system parameters need to be optimized with respect to the envisaged modes and goals of a given application. Rapid action requires a high permeation capability; in order to achieve slow drug release, it is advantageous to ensure gradual penetration through the permeability-barrier and a correspondingly 'finely adjusted' membrane permeability; in order to reach deep regions, high doses are needed; in order to obtain a broad distribution, it is recommended to use carrier concentrations which are not too high.

This application describes some relevant properties of the transfersomes as carriers for the lipid vesicles. Most of the examples pertain to carriers made of phospholipids, but the general validity of conclusions is not restricted to this carrier or molecule class. The vesicle examples should only illustrate the requirements which should be fulfilled in ord r to attain penetration through permeability barriers, such as skin. Similar properties, moreover, ensure carrier transport

across animal or human epidermis, mucosa, plant cuticle, inorganic membranes, etc.

The fact that the cells in a horny skin layer continuously merge with the watery compartments of subcutis is probably one reason for the spontaneous permeation of transfersomes through the 'pores' in this layer: during the permeation process transfersomes are propelled by the osmotic pressure. As an alternative, external pressures, such as an electroosmotic or hydrostatic pressure, however, can also be applied in addition.

Depending on the vesicle dose used, the dermally applied carrier particles can penetrate as deep as the subcutaneous layer. Agents can then be locally released, enriched in (the depth of) the application site, or forwarded to other tissues and body systems through a system of blood and lymph vessels, the precise drug fate being dependent on the carrier size, composition and formulation.

It is sometimes convenient to adjust the pH-value of a formulation immediately after it has been prepared or directly prior to an application. Such an adjustment should prevent the deterioration of individual system components and/or drug carriers under the conditions of initial pH; simultaneously, a physiological compatibility should be achieved. For the neutralization of carrier suspensions, physiologically tolerable acids or bases are most frequently used as well as buffers with a pH-value between 3-12, preferably 5 to 9 and most often 6-8, depending on the goal and site of application. Physiologically acceptable acids are, for example, diluted aqueous solutions of mineral acids, such as hydrochloric acid, sulfuric acid, or phosphoric acid, or organic acids, such as carboxyalkane acids, e.g. acetic acid. Physiologically acceptable bases are, for example, diluted sodium hydroxid ,

suitably ionized phosphoric acids, etc.

Formulation temperature is normally chosen to be well suited for the given substances; for aqueous preparations it is normally in the range of 0 to 95°C. Whenever possible, one should work in the temperature range 18-70°C; particularly preferred are temperatures between 15 and 55°C for the work with fluid chain lipids; the preferred temperature range for the lipids with ordered chains is from 45 to 60°C. Other temperature ranges are possible, however, most notably for the non-aqueous systems or preparations containing cryo- or heat-stabilizers.

If required by the sensitivity of one of the system components, transfersome formulations can be stored in cold (e.g. at 4°C). It is, moreover, possible to make and keep them under an inert atmosphere, e.g. under nitrogen. Shelf-life, furthermore, can be extended if no substances with multiple bonds are used, and if the formulation is (freeze) dried, or if a kit of dry starting materials is dissolved or suspended and processed at the site of application only.

In the majority of cases, carriers are applied at room temperature. But applications at lower or higher temperatures are also possible, especially when synthetic substances are used.

Transfersomal preparations can be processed previously or at the site of application, as has been described, for example, in our previous German patent application P 40 26 833.0-43, and exemplified in several cases in the handbook on 'Liposomes' (Gregoriadis, G., Edits. CRC Press, Boca Raton, Fl., Vols 1-3, 1987), in the monography 'Liposomes as drug carriers' (Gregoriadis, G., Edits. John Wiley & Sons, New York, 1988), or in the laboratory manual 'Liposomes. A Practical Approach' (New, R., Oxford-Press, 1989). If

required any suspension of drugs, moreover, can be diluted or concentrated (e.g. by per ultracentrifugation or ultrafiltration) immediately prior to a final application; additives can also be given into a preparation at this or a previous time. Upon any such manipulation, however, a possible shift of the permeation optimum for a given carrier preparation must be taken into account or prevented.

Transfersomes as described in this applications are well suited to be used as carriers of lipophilic substances, such as fat-soluble biological agents, therapeutics, poisons, etc. But it is quite likely that transfersomes used in combination with water soluble substances, especially when the molecular weight of the latter exceeds 1000 Dt, will be of even greater practical value.

Transfersomes, moreover, can contribute to the stabilization of substances which are sensitive to hydrolysis; they can improve carrier and drug distribution in the specimen and at the site of application and can also ensure a more favourable effect of the drug in time. Basic carrier ingredients can also bring advantages of their own. However, the most important carrier characteristics is the capability of transporting materials into and through a permeability barrier; this opens up a way for applications which prior to this discovery were not feasible.

The specific formulations as described in this invention have been optimized for the topical use on - or in the vicinity of - (a) permeability barrier(s). Particularly interesting barriers of this kind are skin and plant cuticle. (But formulations according to this invention are also well suited for the peroral (p.o) or parenteral (i.v. i.m. or i.p.) application, especially when edge active substances have been chosen in order to keep the drug loss at the site of

application low.) Edge active substances which have a diminished activity, are degraded preferentially, are absorbed particularly efficiently or are diluted strongly at the site of application are especially valuable in this last respect.

In dermatology, application doses of up to 50, often up to 10 and very frequently less than 2.5 (or even less than 1 mg) of carrier substance are used per cm^2 of skin surface, the given masses pertaining to the basic carrier substance. The optimal mass depends on the carrier composition, desired penetration depth and duration of action, as well as on the detailed application site. Application doses useful in agrotechnics are typically lower and frequently below 0.1g pro m^2.

Depending on the goal of application, each formulation can also contain suitable solvents up to a total concentration which is determined by certain plausible physical (no solubilization or appreciable shift of penetration optimum), chemical (no lowering of stability), or biological and physiological (little adversary side effects) formulation requirements.

Quite suitable for this purpose are, for example, the unsubstituted or substituted, e.g. halogenated, aliphatic, cycloaliphatic, aromatic or aromatic-aliphatic hydrocarbons, such as benzol, toluol, methylene chloride or chloroform, alcohols, such as methanol or ethanol, propanediol, erithritol, short-chain alkane carboxylic acid esters, such as acetic acid acid alkylesters, such as diethylether, dioxan or tetrahydrofuran, or mixtures therof.

A survey of the lipids and phospholipids which can be used for the applications as described in this report in addition to the ones already mentioned is given, for example, in 'Form and

function of phospholipids' (Ansell & Hawthorne & Dawson, eds.), 'An Introduction to the Chemistry and Biochemistry of Fatty Acids and Their Glycerides' of Gunstone and in other reference books. All implicitly and explicitly mentioned lipids and surfactants as well as other suitable edge active substances and their preparation are well known. A survey of available surfactants, together with the trademarks under which they are marketed by their manufacturers, is given in the annals 'Mc Cutcheon's, Emulsifiers & Detergents', Manufacturing Confectioner Publishing Co. An up-to-date compilation of the pharmaceutically acceptable agents is given, for example, in 'Deutsches Arzneibuch' (and in the annually updated list 'Rote Liste'); furthermore, in the British Pharmaceutical Codex, European Pharmacopoeia, Farmacopoeia Ufficiale della Repubblica Italiana, Japanese Pharmacopoeia, Nederlandse Pharmacopoeia, Pharmacopoeia Helvetica, Pharmacopée Française, The United States Pharmacopoeia, The United States NF, etc. A concise list of suitable enzymes can be found in the volume on 'Enzymes', 3rd Edition (M. Dixon and E.C. Webb, Academic Press, San Diego, 1979); more recent developments are described in the series 'Methods in Enzymology'. Many examples of the glycohydrate-binding proteins which could be interesting for the use in combination with carriers as described in this invention are quoted in 'The Lectins: Properties, Functions, and Applications in Biology and Medicine' (I.E. Liener, N. Sharon, I.T. Goldstein, Eds. Academic Press, Orlando, 1986) as well as in the corresponding special publications; substances which are particularly interesting for agrotechnical applications are described, for example, in 'The Pesticide Manual' (C.R. Worthing, S.B. Walker, Eds. British Crop Protection Council, Worcestershire, Englande, 1986, e.g. 8th edition) and in 'Wirkstoffe in Pflanzenschutz und Schädlingsbekämpfung', which is published by Industrie-Verband Agrar (Frankfurt); most commonly available antibodies are listed in the catalogue

'Linscott's Directory', the most important neuropeptides in 'Brain Peptides' (D.T. Krieger, M.J. Brownstein, J.B. Martin, Eds. John Wiley, New York, 1983), corresponding supplementary volumes (e.g. 1987) and other special journals.

Methods for the preparation of liposomes, which in the majority of cases can also be used for manufacturing transfersomes, are described, for example, in 'Liposome Technology' (Gregoriadis, Ed., CRC Press) or older books dealing with similar topics, such as 'Liposomes in Immunobiology' (Tom & Six, Eds., Elsevier), 'Liposomes in Biological Systems' (Gregoriadis & Allison, Eds., Willey), 'Targeting of Drugs' (Gregoriadis & Senior & Trouet, Plenum), etc. Corresponding patent publications also are a valuable source of relevant information.

The following examples are aimed at illustrating this invention without restricting it. All temperatures are in degrees Celsius, carrier sizes in nanometers, pressures in Pascal and other units in standard SI system.

Ratios and percentages are given in moles, unless otherwise stated.

Examples 1-13:

Composition:

250-372 mg	phosphatidylcholine from soy-bean (+95 % = PC)
187-34.9 mg	oleic acid (+99 %)
0.312-0.465 ml	ethanol, absolute
10 mM	Hepes

Preparation:

Increasing amounts of oleic acid were pipetted into different volumes of alcoholic PC-solutions containing 75 micromoles of lipid so as to create a concentration series with a lipid/surfactant ratio beginning with L/S=0.5 and increasing by 0.2 units in each step. Subsequently, each lipid sample was supplemented with 4.5 ml of sterile buffer solution and the mixtures were incubated at 4°C for one day. When the pH value had to be adjusted by addition of 1 M NaOH, the first incubation period was followed by another incubation for 24 hours. In order to obtain a final liposome suspension, each sample was thoroughly mixed and filtered through a polycarbonate filter (0.45 micrometer) into a glass vial which was then kept closed at 4°C.

Characterization:

Permeation resistance is assumed to be proportional to the relative pressure needed to perform a secondary filtration through a 0.2 micrometer filter. In this report this resistance is given in relative units of 1 to 10.

Vesicle size is measured by means of dynamic light scattering at 33 degrees C, using a Malvern Zeta-Sizer instrument. For the analysis of correlation curves, a special variant of the software package "Contin" is employed.

In this experimental series all vesicle sizes are relatively independent of the total concentration of edge active substances, in the range of 300 through 350 nm.

Permeation:

Permeation resistance first increases with decreasing relative concentration of fatty acid in the transfersomes. This trend is not monotonous, however. At a lipid/surfactant-ratio of

approx. 2, the liposome permeation capacity starts to increase; but it then decreases again until, for L/S above 3, the transfersomes have nearly lost their capability for passing through narrow constrictions. Vesicles with a lipid/surfactant molar ratio of 1/2 are nearly perfectly permeable, however. (A suspension with 8 % lipid in such case can be filtered nearly as easily as pure water.). At this concentration ratio, which corresponds roughly to 30 % of the solubilization dose of fatty acids in an alkaline suspension, liposomes thus appear to correspond to optimal transfersomes.

Specific data points (0) are shown in figure 1. Vesicles diameters were always measured after permeation experiments.

Examples 14-20:

Composition:

349-358 mg	phosphatidylcholine from soy-bean (+95 % = PC)
63.6-52.2 mg	oleic acid (+99 %)
10 mM	Hepes

Preparation:

4.5 ml of buffer in each case are pipetted to a corresponding amount of lipids and fatty acids to create a concentration series with L/S = 1.92 through 2.4 in the steps of 0.08 units each; the pH value is set to 7.2-7.3 by 1 M NaOH. Lipid suspension after an incubation for 6 days at 4°C is treated by ultrasonication until vesicles with an average diameter of 0.8 micrometers are formed.

Permeation and Characterization:

Permeation resistance is determined as described in examples

1-13. Its value, as a function of the concentration of edge active substance in the system resembles the results of measurements 1-13. The resulting vesicles are somewhat larger than in the previous set of experiments, however, having diameters in the order of 500 nm. This can be explained by the relatively slow material flow during filtration.

Corresponding measured points are shown as (+) in figure 1.

Examples 21-31:

Composition:

322.6-372 mg	phosphatidycholine from soy-bean (+95 %=PC)
96.8-34.9 mg	oleic acid (+99 %)
0.403-0.465 ml	ethanol, absolute
10 mM	Hepes
130 mM	NaCl, p.a.

Preparation:

Preparation procedure used essentially corresponds to the one of examples 14-20. The main difference is that the electrolyte concentration in the present case was isotonic with blood.

Permeation and Characterization:

The measured permeation resistance corresponds, within the limits of experimental error, to the results given in examples 1-13. Vesicle sizes are also similar in both cases. Immediately after the lipid vesicle have been formulated, their diamet rs are in the range of 320-340 nm. 8 days later, however, the vesicle size has increased to approx. 440 nm.

Corresponding experimental data is given in figure 2.

Examples 32-39:

Composition:

184.5-199.8 mg	phosphatidylcholine from soy-bean (+95%=PC)
20.5-22.2 mg	phosphatidylglycerol from egg PC (puriss., Na-salt, =PG)
44.9-26.1 µl	oleic acid (+99 %)
0.165-0.178 ml	ethanol, absolute
4.5 ml	Hepes, 10 mM

Preparation:

Anhydrous PG is mixed with an alcoholic solution of PC to give a clear solution with 90 % PC and 10 % PG. Oleic acid is added to this solution; the resulting lipid/surfactant ratios are between 1.6 and 2.8; an isomolar specimen is made in addition to this. All mixtures are suspended in 4.5 ml of a sterile buffer solution to yield a final lipid concentration of 4 % and then left for 3 days, after a pH-value adjustment with NaOH, in order to age.

Permeation and Carrier Characteristics:

For determining the permeation resistance, the same procedure as in examples 1-13 is used. All measured values are, as a rule, smaller than in the case of carriers which contained no charged species but had a similar L/S-ratio. Based on our experiments with a 4 % suspension of PC and oleic acid we conclude that the relatively low total lipid concentration plays only a minor role in this respect.

As in previous examples, a resistance minimum is observed for

the 4 % PC/PG mixtures; this minimum, however, is found with L/S-ratios which are by some 20 % higher than those measured with 8 % lipid suspensions. Vesicle diameters, however, hardly differ from those measured in examples 1-13.

Precise permeation data is shown in figure 3. All quoted diameters were measured immediately after individual permeation experiments. But even 40 days later, they are hardly bigger than at the beginning; figure 4 illustrates this.

Examples 40-49:

Composition:

301.3-335.4 mg	phosphatidylcholine from soy-bean (+95%=PC)
123.3-80.8 µl	Tween 80 (puriss.)
0.38-0.42 ml	ethanol, absolute
4.5 ml	phosphate buffer, isotonic, sterile

Preparation:

Increasing volumes of Tween 80 are pipetted into appropriate volumes of an alcoholic PC solution. This gives rise to a concentration series with 12.5 through 25 mol-% surfactant (L/S = 4-8). In addition to this, samples with L/S=2 and 3 are also made. After the addition of buffer, lipid vesicles are formed spontaneously: prior to further use, these are made somewhat smaller, with the aid of a 0.8 micrometer filter.

Permeation and Carrier Characteristics:

Permeation resistance is determined in the previously described manner. The corresponding values (0) are shown in the left part of figure 5. As in the case of transfersomes

which contain oleic acid, a region of anomalously high permeation capability (at L/S = 6) can be seen relatively far away from the solubilization point. But it is not before below L/S=4 that a maximum permeability is observed. The transfersomal optimum thus is located in a range which differs by a factor of 1.5-2 from the solubilization point.

Precise permeation data is given in figure 5 (wide lines, left panel). The experimental data in right panel documents the vesicle diameters determined after permeability measurements.

Examples 50-61:

Composition:

314.2-335.4 mg	soy-bean phosphatidylcholine (+95 % = PC)
107.2-80.8 µl	Tween 80 (puriss.)
4.5 ml	phosphate buffer, isotonic, sterile

Preparation:

First Tween 80 and subsequently phosphate buffer are added to appropriate quantities of PC. The resulting mixture is agitated at room temperature for 4 days. The further procedure is as described in examples 40-49.

Permeation and Carrier Characteristics:

Corresponding permeability data is given in figure 5 (thin lines). It confirms, by and large, the results of experiments nos. 40-49.

Examples 62-75:

Composition:

193-361 mg	phosphatidylcholine from soy-bean (grade I,S100)
207.2-38.8 mg	Na-cholate, puriss.
4.5 ml	phosphate buffer (isotonic with a physiologic solution)
	ethanol, absolute

Preparation:

0.5 ml of a hot solution of S100 in ethanol (2/1, M/V) are mixed with sufficient amounts of bile acid salts which give rise to a concentration series with increasing lipid/surfactant ratio between 1/2 and 5/1. The final total lipid concentration is 8 % in all cases.

Vesicle permeation through constrictions and vesicle solubilization:

The permeation resistance of each sample is measured as in examples 1-13. The vesicle size is determined by means of light scattering. (Radii of particles smaller than 5 nm cannot be measured owing to the insufficient power of the laser source used.)

Corresponding measured data is shown in figure 6. It indicates that the permeation resistance of transfersomes with an L/S ratio below 3.5/1 is very small but that this resistance increases significantly at higher L/S values (left panel); the increase of the mean vesicle diameter above L/S = 2.75 (right panel) is probably a consequence of the decreased flow (and thus of a diminished hydrodynamic shear) caused by the greater permeability resistance in this concentration range.

Within only a few hours after preparation the size of vesicles

just above the solubilization limit (at L/S between 1.25/1 and 2.5/1) is significantly bigger than in the vicinity the 'transfersome optimum'. Such undesired consequences of surfactant activity (cf. Fromherz, P. in: 'Galstone disease, Pathophysiology and Therapeutic Approaches', pp. 27-33, Springer, Berlin, 1990) should always be taken into account. At L/S of approx. 1.25/1, solubilization sets in which leads to the formation of, in our case unmeasurably, small mixed micelles of a size of approximately 5 nm.

Examples 76-91:

Composition:

1.627-0.5442 g	phosphatidylcholine from soy-bean (gradeI,S100)
4.373-0.468 g	Na-cholate, puriss.
60 ml	phosphate buffer (physiological)

Preparation:

A 10 % suspension of S100 in phosphate buffer is ultrasonicated at room temperature until the mean vesicle size is approx. 350 nm.

This suspension is divided into three equal volume parts containing 10 %, 1 % and 0.2 % phospholipids. Starting with these preparations, aliquots containing 5 ml of suspension each are prepared. These are supplemented with increasing amounts of sodium cholate (partly in the form of a concentrated micelle suspension), yielding a concentration series with L/S ratios between 1/5 and 5/1. Prior to each permeation- and solubilization measurement, the starting suspension is aged for 1 week at 4°C.

Vesicle permeation through constrictions and vesicle

solubilization:

In order to determine the permeation resistance of these samples two different procedures are used.

In the first series, each suspension is diluted prior to an actual measurement to get a final lipid concentration of 0.2 %; subsequently it is pressed through a filter with a pore size of 0.1 micrometers. The sample resistance is identified with the inverse value of the volume which has passed through the filter pores during a period of 5 minutes.

In the second series, the permeation resistance is determined as in examples 1-13 and finally renormalized by dividing the values thus obtained with regard to the final lipid concentration.

The resulting data shows that both the solubilization limit and the position of a 'transfersome optimum' expressed in terms of preferred L/S ratios are dependent on the overall lipid concentration. In the case of a 10 % suspension the corresponding values are approx. 1/1 and 2.75/1, respectively; for the 0.2 % suspension they increase to 1/4 and 1/1, however.

Examples 92-98:

Composition:

16.3-5.4 mg	phosphatidylcholine from soy-bean (Grade I, S100)
41.5-5.5 mg	Na-desoxycholate, puriss.
5 ml	phosphate buffer (physiological)

Preparation:

A suspension of 1 % desoxycholate containing vesicles is prepared as described in examples 76-91.

Vesicle permeation through constrictions and vesicle solubilization:

The measurements of this experimental series show that vesicles containing desoxycholate are solubilized already at L/S ratios near 1/2, i.e. at an L/S ratio which is by a factor of 2-3 lower than in the case of S100/Na-cholate vesicles.

Examples 99-107:

Composition:

3 mM Suspension of phosphatidylcholine from soy-bean (grade I, S100) in phosphate buffer Na-cholate, puriss.

Preparation:

A 3 mM suspension of S100 in phosphate buffer is partly homogenized at room temperature. 3 ml of this suspension are supplemented each with increasing amounts of sodium cholate in order to create a series with increasing L/S ratios between 1/2 and 12/1. After three days of incubation, these aliquots are ultrasonicated at 55°C, using a 50 % duty-cycle; simultaneously, the optical density at 400 nm of each sample is recorded. An analysis of the resulting experimental data within the framework of a bimodal exponential model reveals two characteristic vesicularization rates (tau 1 and tau 2); these characterize the temporal dependence of the number of lamellae in each vesicle (tau 1) and the changes in the mean size of vesicles (tau 2).

Vesicle characterization and deformability.

The tau 1 and tau 2 values represented in figure 7 show that the mechanical properties of transfersomes, which are reflected in the value of parameter tau 2, exhibit a similar L/S dependence as the solubilization and permeation tendency (cf. fig. 6). For a 0.2 % suspension investigated in this series 1 cholate molecule per lipid is required for a rapid formation of vesicles (for the formation of largely unilamellar vesicles).

Examples 108-119:

Composition:

121.2-418.3 mg	phosphatidylcholine from soy-bean (Grade I,
PC) 378.8-81.7 mg	Triton X-100
4.5 ml	0.9 % NaCl solution in water

Preparation:

A 10 % PC-suspension in isotonic solution of sodium chloride is homogenized at 22°C until the mean size of lipid vesicles is approx. 400 nm. This suspension is then distributed in aliquots of approx. 4.8 ml. A sufficient volume of Triton X-100 is pipetted into each of these aliquots to give a concentration series with nominal PC/Triton ratios in the range of 0.25 through 4 in steps of 0.5. All resulting samples are occasionally mixed and incubated at 4°C for 14 days.

Vesicle solubilization

The optical density (OD (400 nm)) of a lipid-triton mixture after a 10-fold dilution provides insight into the vesicle

solubilization; this is represented in the right panel of figure 8. The solubilization limit is approx. 2 triton molecules per PC-molecule. Right below this limit, the optical density (OD (400 nm)) - and thus the vesicle diameters - attain the greatest values. At PC/triton ratios higher than 2,5/1, the change in the optical density of given suspensions is only minimal.

Vesicle permeation and characteristics:

In order to evaluate the permeation capability of the resulting lipid vesicles and transfersomes all suspensions were pressed through fine-pore filters (0.22 micrometer), as described in examples 1-13. The required pressure increases gradually with the decreasing total triton concentration in the suspension; for L/S ratios higher than 2/1 this significantly limits the permeation capability of carriers.

Corresponding results are summarized in the left half of figure 8.

Examples 120-128:

Composition:

403,5-463,1 mg	dipalmitoyl tartaric acid ester, Na-salt
96,5-36,9 mg	laurylsulfate, Na-salt (SDS)
4,5 ml	triethanolamine buffer, pH 7.5

Preparation:

In this test series a synthetic lipid, which is not found in biological systems, was chosen to be the basic transfersome constituent. For each experiment the required dry lipid mass was weighed into a glass vial and mixed with 4.5 ml of buffer.

The latter contained sufficient amounts of sodiumdodecylsulfate (SDS) to give various L/S ratios between 2/1 and 6/1. Well mixed suspensions were first kept at room temperature for 24 hours and subsequently mixed again thoroughly.

Permeation capacity and vesicle characteristics:

Liposomes were pressed through a 0.2 micrometer filter. Simultaneously, the permeation resistance was measured. Vesicles with an L/S ratio below 4/1 can pass the membrane pores very easily; in contrast to this, all vesicles with lower surfactant concentrations or vesicles without edge active components can pass through the porous constrictions only with difficulty (not before an excess pressure of 5 MPa has been created) or not at all (membranes burst).

Examples 129-136:

Composition:

101,6-227 mg	phosphatidylcholine from soy-bean
148,4-22,2 mg	octyl-glucopyranoside (ß-octylglucoside),
puriss. 9,85 ml	phosphate buffer, pH 7,3
	ethanol, absolute

Preparation:

Phosphatidylcholine in ethanol (50 %) and octyl-glucopyranoside were mixed in different relative ratios in order to prepare a concentration series with increasing L/S values between 1/4 and 2/1 (and a final total lipid concentration of 2.5 %). Each lipid mixture in a glass vial was then supplemented with 4.5 ml of buffer. Subsequently, the resulting suspension was mixed in an agitator for 48 hours

at 25°C. The suspension turbidity was greater for the specimen containing lower amounts of octylglucoside. A fine sediment formed in standing samples. Each suspension was mixed thoroughly before the experiment.

Vesicle permeation and characteristics:

All suspensions can be filtered without any problem through filters with a pore diameter of 0.22 micrometer, using only minimal excess pressures of less than 0.1-0.2 MPa; the only two exceptions are the samples with the lowest surfactant concentration. These give rise to small permeation resistances which on the renormalized scale (cf. figures 1-5) corresponds to values of approx. 1 and 2.5, respectively. Figure 9 presents said data.

If the pore diameter is reduced to 0.05 micrometers only suspensions with L/S ratios below 2/1 can still be filtered.

Irrespective of the pore size used all preparations with L/S ratios below 2/1 are unstable; after only a few days, a phase separation is observed between a micelle rich and a vesicle rich phase.

Examples 137-138:

Composition:

43,3 mg, 50 mg	phosphatidylcholine from soy-bean
0.5 mg	phosphatidylethanolamine-N-fluorescein
6,7 mg, 0 mg	cholate, Na-salt, p.a.
5 ml	Hepes-buffer, pH 7,3

Preparation:

Phosphatidylcholine with the addition of 1 %-fluoresceinated lipids with or without desoxycholate is suspended in 5 ml buffer. The lipid/surfactant ratio is 3.5/1 or 1/0. Both 1 %-suspensions are then ultrasonicated in a glass vial for 1.5 or 15 minutes (25 W, 20°C), until the mean vesicle size is approx. 100 nm.

Spontaneous vesicle permeation:

Onto a Millipore-filter with 0.3 micrometer pore diameter, mounted into a Swinney-holder, the lower half of which has been wetted and filled with water, 50 microliters of a lipid suspension are pipetted through the upper opening. By a gentle swinging motion, a relatively homogeneous sample distribution on the filter surface is ensured. After 30 minutes, the holder is carefully opened and left to dry for 60 minutes. Subsequently the water from below the filter is collected and checked fluorimetrically (excitation 490 nm, emission 590 nm). (The determined light intensity is a measure of the permeation capacity.)

The transport of fluorescence markers mediated by surfactants containing transfersomes gives rise to a fluorescence signal of 89.5; in control experiment a value of 44.1 is established. This indicates that transfersomes are capable of transporting encapsulated substances across permeability barriers.

Examples 137-139:

Composition:

43,5, 45,3, 50 mg	phosphatidylcholine from soy-bean
0.5 mg	phosphatidylethanolamine-N-fluorescein
6,5, 4,7, 0 mg	desoxycholate, Na-salt, p.a.

25 ml	Hepes-buffer, pH 7,3

Preparation and results:

Lipid vesicles are made and tested as described in examples 137-138. Measurements show that the transfersomes which contain deoxycholate already show similarly good results at a characteristic L/S ratio of 5/1 as transfersomes which contain cholate at a ratio of L/S=3.5.

Examples 140-142:

Composition:

50 mg; 43,3 mg; 15,9 mg	phosphatidylcholine from soy-bean
0.5 mg	phosphatidylethanolamine-N-fluorescein
0 mg; 6,7 mg; 34,1 mg	cholate, Na-salt, p.a.
5 ml	Hepes-buffer, pH 7,3

Preparation:

Lipid vesicles consisting of phosphatidylcholine and a fluorescent additive were made as in examples 137-138. For this experiment, suspensions with a lipid/surfactant ratio of 1/0, 4/1 and 1/4 were used. The former two contained fluorescent lipid vesicles, the latter a micellar suspension.

Spontaneous penetration into plant leaves:

A fresh onion is carefully opened in order to gain access to individual leaves; these correspond to low-chlorophyll plant leaves. For each measurement, 25 microliters of a fluorescinated suspension are applied onto the concave (inner or upper) side of each onion leaf; as a result of this a

convex droplet with an area of approx. 0.25 square centimeters is formed. (Carriers which contain surfactants can be easily identified owing to their higher wetting capability.) After 90 minutes the (macroscopically) dry lipid film is eliminated with the aid of a water stream from a jet-bottle with a volume of 50 ml.

After this treatment, the 'leaf surface' attains a slightly reddish appearance in the case of surfactant containing transfersomes as well as mixed micelles. Leaves incubated with surfactant-free vesicles cannot be distinguished from the untreated leaves.

Fluorescence measurements using a red filter (excitation through a blue filter from above) show that leaves which were covered with transfersomes are intensively fluorescent throughout the treated area. In certain places extremely brilliant aggregates are detected; these probably correspond to the non-eliminated vesicle-clusters. The fluorescence of leaves which were treated with a surfactant solution in some places is comparably intensive; at other positions their fluorescence is weaker, however, than in the case of transfersome-treated leaves.

The leaves which were treated with standard lipid vesicles do not fluoresce. Over large surface areas they are indistinguishable from the non-treated leaf regions.

This shows that transfersomes can transfer lipophilic substances spontaneously and irreversibly into a plant leaf or its surface. Their penetration capacity exceeds that of preparations containing highly concentrated surfactants, i.e. well established 'membrane fluidizers'.

Examples 143-145:

Composition:

50 mg; 43,5mg; 17,1 mg	phosphatidylcholine from soy-bean
0.5 mg	phosphatidylethanolamine-N-fluorescein
0 mg; 4,7 mg; 32,9 mg	desoxycholate, Na-salt, p.a.
5 ml	Hepes-buffer, pH 7,3

Preparation and results:

The preparation and results are identical with those of experiments 140-142.

Examples 146-148:

Composition:

50 mg; 36,4; 20 mg	phosphatidylcholine from soy-bean
0.5 mg	phosphatidylethanolamine-N-fluorescein
0 mg; 13,6 mg; 30 mg	Brij 35
5 ml	Water

Preparation and results:

Preparation and results are comparable to those of experiments 140-142 and 143-145.

Examples 146-150:

Composition:

84,2 to 25 mg	phosphatidylcholine from soy-bean 80 %
75 kBq	Giberellin A4, 3H-labelled
15,8 to 75 mg	polyoxyethylene (23)-laurylether (Brij 35)
1 ml	water

ethanol, absolute

Preparation:

An ethanolic lipid solution (50 %) is mixed with a corresponding amount of an ethanolic solution of giberellin and suspended in 1 ml of water or in appropriate volumes of a surfactant suspension to obtain a total lipid concentration of 10 % and L/S ratios of 8/1, 4/1, 2/1, 1/1 and 1/2. The resulting (mixed) suspension is then briefly homogenized with the aid of ultrasound so that the mean vesicle size is always below 300 nm.

Carrier suspensions are distributed over the surface of 3 leaves of Ficus Benjaminii; there, they are permitted to dry for 6 hours. After subsequent intensive washing of each leaf surface with 5 ml of water per square centimetre and destaining with a peroxide solution, the radioactivity in the homogenized plant material is measured scintigraphically in a beta-counter.

Agent transport in plant leaves:

Experiments show, as in examples 140-142, that transfersomes can bring the agent molecules into a leaf surface much more effectively than a micellar solution.

Examples 151-157:

Composition:

32,8-0.64 mg	phosphatidylcholine from soy-bean (purity higher than 95 %, PC)
75 kBq	dipalmitoylphosphatidylcholine tritium-labelled

2,2-34,4 mg	bile acid, Na-salt, p.a.
0.32 ml	phosphate buffer, pH 7,3

Preparation:

In each case, 35 mg of lipid are mixed with tritium-labelled dipalmitoylphosphatidylcholine in chloroform. After thorough drying under vacuum, the resulting mixture is suspended in 0.32 ml of buffer; the nominal surfactant/lipid ratios are 0; 0.125; 0.167; 0.263; 0.5 and 1 mol/mol. All suspensions are ultrasonicated until they are comparably opalescent, with the exception of the last, optically clear micellar solution. (The time for efficient necessary sonication decreases with increasing S/L). Control measurements with non-radioactive suspensions indicate that the mean 'particle' size in all samples must be around 100 nm. In all experiments approximately 1 day old suspensions are used.

Penetration into and through the intact skin:

On the back of an immobilized nude-mouse anaesthesized with ether six areas of 1x1 cm are marked. Each of these areas is covered with 20 microliters of a carrier suspension at 3x5 minutes intervals. 60 minutes later, the mouse is killed. From each treated area a sample is excised which is then cut to pieces, solubilized and de-stained. The skin-associated radioactivity is measured scintigraphically.

The corresponding results are summarized in figure 10. For comparison, the normalized values are also given which were taken from our patent application pertaining to the use of liposomes for topical anaesthesia. Optimal transfersomes are appreciably better than non-optimal preparations containing surfactants.

Examples 158-162:

Composition:

31 mg	phosphatidylcholine from soy-bean (purity higher than 95 %, PC)
75 kBq	dipalmitoylphosphatidylcholine tritium-labelled
4 mg	deoxycholate, Na-salt, p.a.
0.32 ml	phosphate buffer, pH 7,3

Preparation:

In each case 35 mg of lipid (PC and deoxycholate) are mixed with tritium-labelled dipalmitoylphosphatidylcholine in a chloroform solution. The resulting lipid mixture is dried and then dissolved in 30 microliters of warm, absolute ethanol. This solution is then mixed with 0.32 ml of a buffer solution (phosphate buffer, 10 mM, 0.9 % NaCl); this corresponds to a lipid/surfactant ratio of 4/1. The resulting suspension is thoroughly mixed and subsequently filtered through filters with pore sizes of 0.8; 0.45; 0.22 and 0.1 micrometers; this gives rise to vesicles with diameters of approx. 800, 400, 200 or 100 nm (suspensions A, B, C, D).

Penetration into and through the skin:

Tails of 2 anaesthesized mice are treated with 50 microlitres of a corresponding vesicle suspension for 15 minutes. Two control animals obtain an i.v. injection of 0.2 ml 1/10 diluted suspension B. After 30, 60, 120, 180, 240 and 360 minutes, blood specimens are drawn from the tail-tip. The radioactivity of these samples, which is determined by means of beta-scintigraphy, is a reliable indication of the systemic concentration of carrier-associated, radioactively labelled

lipids.

Experimental data show (fig. 11) that systemically applied transfersomes are eliminated from blood comparably as rapidly as standard liposomes. The size of carrier particles appears not to affect the spontaneous penetration into skin. All transfersomes investigated in this study can penetrate intact skin and get into the depth of a body quite effectively within a period of 4 hours at approx. 1 carrier; tendency increasing.

Examples 163-165:

Composition:

88 mg	phosphatidylcholine from soy-bean (purity higher than 95 %, PC)
75 kBq	inulin, tritium labelled
12 mg	deoxycholate, Na-salt, p.a.
100 ml	ethanol, absolute
0.9 ml	isotonic salt solution

Preparation:

100 mg of PC dissolved in 100 ml of warm ethanol, or a corresponding PC/deoxycholate solution (L/S = 4.5), are mixed with 0.9 ml of an isotonic salt solution (suspensions A and B, respectively). Each suspension is ultrasonicated until the mean vesicle size is about 150 nm.

12 microlitres of an aqueous solution of tritium-labelled inulin are pipetted into 38 microliters of a freshly prepared suspension of empty liposomes (A) or transfersomes (B). Subsequently, all mixtures are sonicated in closed vials for 60 minutes in an ultrasound bath at room temperature; they are all used for experiments within 24 after vesicle preparation.

Spontaneous inulin transfer through the skin:

On the abdomen of NMRI-mice in general anaesthesia, which three days before were depillated using medical tweezers, 10 microlitres of a vesicle suspension containing inulin in every case are applied twice at time intervals of approx. 3-5 minutes.

15, 30, 60, 120, 180, 240, 300 and 360 minutes later, 0.05 ml of blood are routinely taken from the tail of a each mouse to be then investigated scintigraphically. 6 hours later the subcutaneous tissues at the application site, as well as liver and spleen of all animals of this experiment are collected. After solubilization and decolouring procedures, these organs are also checked scintigraphically.

The results of this study are collected in figure 12. They show that normal liposomes can hardly mediate a percutaneous inulin uptake; in contrast to this, 6 hours later approx. 1.4 % of this marker which was applied in the form of transfersomes are found in the blood. This transfer sets in approximately 2-3 hours after the application and is not yet completed 6 hours after each application.

After 6 hours in the case of transfersomes, an average of 0.8 % (this corresponds to 24.1 % of the recovered dose) are in the skin at the application site; 0.9 % are found in the liver; spleen contains less than 0.1 % of the absolute dose. In the body (blood, spleen, liver) approximately 73.8 % of the recovered dose are thus found again.

In contrast to this, approximately 2 % of the normal liposomes at the application site can be detected by eye, the corresponding doses in the liver and spleen being below

0.1 %. This corresponds to a recovery of 95.3 % at the application site and 6.7 % of this dose in the body of the test animal.

Example 166:

Composition:

386 mg	phosphatidylcholine from soy-bean (purity > 95 %)
58.5 mg	sodium-cholate (L/S = 3,5)
500 µl	ethanol (96 %)
2.25 ml	0.9 % NaCl solution (per inject.)
2.25 ml	Actrapid HM 40 (corresponds to 90 I.U. of recombinant human insulin)

Preparation:

Samples are prepared essentially as described in examples 62-75. A mixture of aqueous salt solution and human recombinant insulin (with 6.75 mg m-cresole) is mixed with a lipid solution in ethanol. The resulting, opaque suspension is aged over night. 12 hours later, this suspension is pressed through a sterile filter (Anodisc, pore diameter 0.2 micrometers) with the aid of nitrogen gas with excess pressure of 0.25 MPa under sterile conditions to be then filled into the glass container.

The nominal lipid/surfactant ratio is 3.5; the calculated molar surfactant concentration in the lipid double layer is approx. 5/1. This corresponds to 50 % of the concentration required for solubilization.

The mean radius of vesicles in final suspension in this experiment was 97 nm.

Application:

0.5 ml of a fresh, insulin containing transfersome suspension are applied onto the untreated skin of the left forearm of an informed, healthy male volunteer aged 37 years (starved for 18 hours) and distributed over an area of approx. 10 cm^2. 5 minutes later, additional 300 microlitres of identical suspension are positioned in two halves on the forearm and upper arm, respectively. 5-10 minutes later, the suspension on the upper arm (dose approx. 2,5 mg/cm^2) has almost completely disappeared; it has thus nearly completely penetrated into skin. In contrast to this, lipids applied onto the forearm (dose approx. 7.5 mg/cm^2) are still well perceptible.

Activity:

In order to assess the biological activity of insulin, approx. 2 hours before the sample application, a permanent, soft catheter is placed into a vein in the right hand. Every 15-45 minutes, 1-1.5 ml of blood are collected from this catheter; the first 0.5-1 ml thereof are discarded; the remaining 0.5 ml are measured with a standard enzymatic glucose test. In each case three determinations with three to four independent specimens are made. The corresponding experimental data is summarized in figure 13. It shows that transfersomes mediate a significant hypoglycemia in the peripheral blood some 90 minutes after the drug application; this effect lasts for approx. 2 hours and amounts to approx. 50 % of the magnitude of the hypoglycemic effect of a comparable dose of subcutaneously applied insulin; the effect of the former lasts 200 % longer, however.

Examples 167-172:

Composition:

956 mg	phosphatidylcholine from soy-bean (+95 %)
0-26 mg	sodium-deoxycholate
1 mg	prostaglandine E1
1 ml	ethanol absolute
50 ml	0.9 % NaCl solution (per inject.)

Preparation:

1 ml of ethanol is pipetted into a glass flask with 1 mg of prostaglandine. After thorough mixing, the resulting prostaglandine solution is transferred to the appropriate amount of dry lipid in another glass vial. The original flask is flushed once again with the new lipid/prostaglandine solution and subsequently supplemented with 6 ml of an isotonic salt solution. The prostaglandine containing flask is washed twice with 2 ml of 0.9 % NaCl and mixed with the original lipid suspension. The sample is then divided into 5 parts; into individual aliquots sodium-desoxycholate is added at concentrations of 0; 1.6; 3.25; 6.5 or twice 13 mg/ml.

The resulting 10 % suspensions are aged for 24 hours. Subsequently they are either ultrasonicated or filtered manually through a 0.2 micrometer-filter, depending on cholate concentration. The specimens with the highest surfactant concentration are either filtered or ultrasonicated. Finally, the samples are diluted to obtain a final PGE1 concentration of 20 micrograms/ml and kept in dark glass bottles in a refrigerator. Vesicle radius right after sample preparation is 85 nm, two months later 100 nm.

Application and Action:

In each experiment 0.25 ml of a lipid suspension are applied on neighbouring but not interconnected regions of abdominal skin. 10 minutes later the skin surface is macroscopically dry; 15 minutes later, some of the application sites show a reddish appearance which, according to the test person's statement, is associated with a weak local pain. The intensity of oedema grades as 0, 0, 0, 0-1, 3 and 3 points (on a scale from 1-10).

This shows that merely transfersomes - but not liposomes or sub-optimal surfactant-containing vesicles - can penetrate into intact skin and thereby transfer drugs into body. The precise mode of sample preparation plays no role in this.

Examples 173-175:

Composition:

79.4 mg; 88.5 mg	phosphatidylcholine from soy-bean (+95%)
20.6 mg, 11.5 mg	sodium-deoxycholate
10 µg	hydrocortison
0.1 ml	ethanol absolute
1 ml	phosphate buffer, physiological

Preparation:

Lipids and hydrocortison are mixed as approx. 50 % ethanolic solution and subsequently supplemented with 0.95 ml of phosphate buffer. The resulting, very heterogeneous suspension is treated with ultrasound (25 W, 3-5 min). Specimens with an L/S ratio of 2/1 can be homogenized with ease, specimens with L/S = 4/1 are relatively difficult to homogenize.

Specimens with 1 and 2.5 weight-% result in stable suspensions

independent of the precise L/S ratio; 10 weight-% of agent cannot be incorporated into stable transfersomes of the given composition.

Examples 175-200:

Composition:

1.1 - 2mg	phosphatidylcholine from soy-bean (+95%=PC)
0 - 32.5 mol-%	Tween 80
pH 7.2	isotonic phosphate buffer

Preparation:

Different amounts of phospholipid and surfactant in each experiment are weighed or pipetted into 25 ml of buffer at ratios which yield suspensions with 0 -32,5 mol-% of Tween 80 and a constant total lipid concentration of 2 %. Specimens are sterilized by filtering, filled into sterile glass vials and aged for 4 through 34 days. Then, the optical density of each sample is determined. This depends strongly on surfactant concentration but hardly on time within the framework of measuring conditions.

Characterization:

23 specimens each containing 3 ml of an individual lipid suspension are ultrasonicated in closed vials in a bath sonicator. Three, four and six hours later the samples' optical density is determined. Such measurements are repeated with every new sample series after the relative sample positions were exchanged in a systematic manner; the determination of optical density, again, is performed three, four and six hours after the start of sonication. All values corresponding to one concentration are summed up and divided

by the number of measurements; the resulting value is a measure of the samples' capacity for vesicularization under given conditions.

This procedure is an alternative or a supplement to the permeation resistance measurements as described in examples 40-49. Figure 16 shows, for example, that the amount of surfactant required for good mechanical deformability in the case of Tween 80 is 2-3 times lower than the corresponding solubilization concentration. This result is in good accord with the results of the permeation experiments.

Examples 201-215

Composition:

256.4-447 mg	phosphatidylcholine from soy-bean (+95% PC)
243.6-53.1 mg	Brij 96
0.26-0.45 ml	ethanol, absolute
4.5 ml	phosphate buffer, pH 6,5, 10 mM

Preparation:

Increasing volumes of Brij 96 are pipetted into the corresponding volumes of an alcoholic PC solution. Thus, a concentration series is obtained with L/S values between 1/1 and 1/8. After the addition of a buffer very heterogeneous liposomes are formed which are homogenized by means of filtering through a 0.2 μm filter.

Permeation and carrier characteristics:

The already described method for the determination of suspensions permeability resistance is used. Corresponding values are given in the left panel of figure 14 as circles or

crosses (two independent test series). The functional dependence of the samples' permeability resistance as a function of the L/S ratio is similar to that of comparable transfersomes and is illustrated in the right panel of figure 14. The maximum permeation capacity is not reached before the L/S-value is below 3.

Examples 216-235

Composition:

202,0-413 mg	phosphatidylcholine from soy-bean (+95%=PC)
298,0-87,0 mg	Myrj 49
0.26-0.45 ml	ethanol, absolute
4.5 ml	phosphate buffer, pH 6,5, 10 mM

Preparation and Characterization:

Transfersomes are made and characterized as described for examples 201-215. Their permeation properties as a function of the relative surfactant concentration in the individual specimen is given in the left panel of figure 15. The right panel gives corresponding equilibrium values; the latter, however, provide no information about vesicle suitability for permeation and agent transport.

Example 236:

Composition:

144,9 mg	phosphatidylcholine from soy-bean
24.8 mg	desoxycholate, Na-salt
1.45 ml	Actrapid HM 100 (145 I.U.)
0.16 ml	ethanol, absolute

Preparation:

Appropriate quantities of both lipids are dissolved in corresponding amounts of ethanol and mixed with a standard solution of insulin. 12 hours later, the crude carrier suspension is homogenized by means of filtration. Average vesicle diameter is 225 ± 61 nm and nominal insulin concentration is 83 I.U. Over an area of appr. 10 square centimeters on the right forearm 0.36 ml (30 I.U.) of insulin in transfersomes are distributed. Blood samples are taken every 10 minutes through a heparinized soft catheter positioned in a vein in the right forearm; the first 0.5 ml are always discarded; the following 0.5-0.8 ml of each sample are sedimented and immediately frozen; the remainder of each sample is used for the determination of blood glucose concentration during the experiment.

Activity:

These liposomes with a relatively high surfactant concentration have only a very limited capability of transporting insulin across skin, as is seen from figure 17. Depending on the choice of data used for evaluation, the lowering of the blood glucose level does not exceed 2 to 5 mg/dl over a period of 30-40 minutes at the most. The effect of a comparable subcutaneous injection is 50 to 200 times higher. Surfactant-containing liposomes, which have not been optimized with regard to their 'transfersomal' properties, are consequently poorly suited for the use as carriers in the case of dermal applications. Surfactant concentration in such carriers thus cannot mediate an optimal agent permeation through skin.

This shows that formulations prepared according to this invention can (still) have a partial activity even if their

content of edge active substances has not been optimized; however, a maximum advantage can only be achieved after the concentration of an edge active substance requiring maximum permeation has been determined and used as described in this patent application.

Possible use of transfersomes for the application of antidiabetics, most notably of insulin, which has been discussed above in examples 166 and 236, will be investigated in more detail in the following text.

Attempts to bring antidiabetic agents into a body without the use of an injection needle have been known for quite some time already (see, for example, the review article by Lassmann-Vague, Diabete. Metab. 14,728,1989). It has been proposed, for example, to use implantable insulin containers (Wang, P.Y, Biomaterials 10. 197, 1989) or pumps (Walter, H et al., Klin. Wochenschr. 67, 583, 1989), to administer an insulin solution transnasally (Mishima et al., J. Pharmacobio.-Dynam. 12, 31, 1989), perocularly (Chiou et al., J. Ocul. Pharmacol. 5, 81, 1989), perorally in a liposomes suspension (Rowland & Woodley, Biosc. Rep. 1, 345, 1981) or transrectally; in order to introduce insulin molecules through the skin, a corresponding solution was jet-injected (Siddiqui & Chies, Crit. Rev. Ther. Drug. Carrier. Syst. 3, 195, 1987), or brought through the skin with the aid of small injectors (Fiskes, Lancet 1, 787, 1989), electric fields (Burnette & Ongpipattanakul, J. Pharm. Sci. 76, 765, 1987; Meyer, B.R et al., Amer. J. Med. Sci. 297, 321, 1989); chemical additives should also support drug permeation.

All these procedures have hardly brought any real improvements for the therapy of diabetes patients - with the exception of jet injection, perhaps; but the latter is only a very refined, technically extremely complicated form of injection and,

consequently, not very common. The daily therapy of each insulin-dependent patient, consequently, still involves injecting an insulin solution under the skin or into the muscle tissue (De Meijer, P. et al., Neth. J Med. 34, 210. 1989).

Lipids have thus far been discussed as excipients for delayed insulin release in insulin implants (Wang, P.Y Int. J Pharm. 54, 223, 1989); in the form of liposomes they were also suggested for use as vehicles for peroral applications (Patel, 1970), without the therapeutic results really being reproducible, however, (Biochem. Int. 16, 983, 1988). Subsequent publications in the field of insulin containing liposomes, therefore, have dealt with methodological rather than therapeutic issues (Wiessner, J. H. and Hwang, K. J. Biochim. Biophys. Acta 689, 490 1982; Sarrach, D. Stud. Biophys. 100. 95, 1984; Sarrach, D. and Lachmann, U. Pharmazie 40. 642, 1985; Weingarten, C. et al., Int. J. Pharm. 26, 251, 1985; Sammins, M.C. et al., J. Pharm. Sci. 75, 838, 1986; Cervato, G. et al., Chem. Phys. lipids 43, 135, 1987).

According to this invention, the transfersomes described above are used for non-invasive applications of antidiabetic agents, most frequently of insulin, in formulations which were optimized for this purpose.

It is advantageous to use at least one carrier substance for this purpose from the class of physiologically tolerable polar or non-polar lipids or some other pharmacologically acceptable amphiphiles; well-suited molecules are characterized by their ability to form stable agent carrying aggregates. The preferred aggregate form are lipid vesicles, the most preferred membrane structure is a lipid double layer.

It is, furthermore, considered advantageous if at least one

such substance is a lipid or a lipoid from a biological source or some corresponding synthetic lipid; or else, a modification of such lipids, for example a glyceride, glycerophospholipid, sphingolipid, isoprenoidlipid, steroid, sterine or sterol, a sulfur- or carbohydrate-containing lipid, or any other lipid which forms stable double layers; for example, a half-protonated fluid fatty acid. Lipids from eggs, soy-bean, coconuts, olives, safflower, sunflower, linseed, whale oil, Nachtkerze or primrose oil, etc. can be used, for example, with natural, partly or completely hydrogenated or exchanged chains. Particularly frequently, the corresponding phosphatidylcholines are used; as well as phosphatidyl-ethanolamine, phosphatidylglycerol, phosphatidylinositol, phosphatidic acids and phosphatidylserines, sphingomyelines or sphingophospholipids, glycosphingolipids (e.g. cerebrosides, ceramidpolyhexosides, sulfateids, sphingoplasmalogenes); gangliosides or other glycolipids are also suitable for the use in transfersomes according to this invention. Amongst the synthetic lipids especially the corresponding dioleoyl-, dilinoleyl-, dilinolenyl-, dilinolenoyl-, diaracidonyl-, dimyristoyl-, less frequently dipalmitoyl-, distearoyl-, phospholipide or the corresponding sphingosin derivatives, glycolipids or other diacyl- or dialkyl-lipids are used; arbitrary combinations of the above-mentioned substances are also useful.

It is advantageous if an edge active substance is a nonionic, a zwitterionic, an anionic or a cationic surfactant. It can also contain an alcohol residue; quite suitable components are long-chain fatty acids or fatty alcohols, alkyl-trimethyl-ammonium-salts, alkylsulfate-salts, cholate-, deoxycholate-, glycodeoxycholate-, taurodeoxycholate-salts, dodecyl-dimethyl-aminoxide, decanoyl- or dodecanoyl-N-methylglucamide (MEGA 10, MEGA 12), N-dodecyl-N,N-dimethylglycine, 3-(hexadecyldimethylammonio)-propanesulfonate, N-hexadecyl-

sulfobetaine, nonaethyleneglycol-octylphenylether, nonaethylene-dodecylether, octaethyleneglycol-isotridecyl-ether, octaethylene-dodecylether, polyethylene glycol-20-sorbitane-monolaurate (Tween 20), polyethylene glycol-20-sorbitane-monooleate (Tween 80), polyhydroxyethylene-cetylstearylether (Cetomacrogo, Cremophor O, Eumulgin, C 1000) polyhydroxyethylene-4-laurylether (Brij 30), polyhydroxyethylene-23-laurylether (Brij 35), polyhydroxyethylene-8-stearate (Myrj 45, Cremophor AP), polyhydroxyethylene-40-stearate (Myrj 52), polyhydroxyethylene-100-stearate (Myrj 59), polyethoxylated castor oil 40 (Cremophor EL), polyethoxylated hydrated castor oil, sorbitane-monolaurate (Arlacel 20, Span 20), especially preferred decanoyl- or dodecanoyl-N-methylglucamide, lauryl- or oleoylsulfate-salts, sodiumdeoxycholate, sodiumglycodeoxycholate, sodiumoleate, sodiumelaidate, sodiumlinoleate, sodiumlaurate, nonaethylene-dodecylether, polethylene-glycol-20-sorbitane-monooleate (Tween 80), polyhydroxyethylene-23-lauryl ether (Brij 35), polyhydroxy-ethylene-40-stearate (Myrj 52), sorbitane-monolaurate (Arlacel 20, Span 20) etc.

Amongst the most suitable surfactants in these classes of substances are: n-tetradecyl(=myristoyl)-glycero-phosphatidic acid, n-hexadecyl-(=palmityl)-glycero-phosphatidic acid, n-octadecyl(=stearyl)-glycero-phosphatidic acid, n-hexadecylene(=palmitoleil)-glycero-phosphatidic acid, n-octadecylene(=oleil)-glycero-phosphatidic acid, n-tetradecyl-glycero-phosphoglycerol, n-hexadecyl-glycero-phosphoglycerol, -n-octadecyl-glycero-phosphoglycerol, n-hexadecylene-glycero-phosphoglycerol, n-octadecylene-glycero-phosphoglycerol, n-tetradecyl-glycero-phosphoserine, n-hexadecyl-glycero-phosphoserine, -n-octadecyl-glycero-phosphoserine, n-hexadecylene-glycero-phosphoserine and n-octadecylene-glycero-phosphoserine.

Total concentration of the basic carrier subtance is normally between 0.1 and 30 weight-%; preferably, concentrations between 0.1 and 15 %, most frequently between 5 and 10 % are used.

Total concentration of the edge active substance in the system amounts to 0.1 % through to 99 mol-% of the quantity which is required to solubilize the carrier, depending on each application. Frequently, the optimum is drug dependent - in a concentration range between 1 and 80 mol-%, in particular between 10 and 60 mol-%; most frequently values between 20 and 50 mol-% are favoured.

The concentration of the drug agent in the case of insulin is most frequently in the range between 1 and 500 I.U./ml; concentrations between 20 and 100 I.U./ml are preferred; carrier concentration in the latter case is in the range between 0.1-20 weight-%, frequently between 0.5 and 15 weight-%, most frequently between 2.5 and 10 weight-%.

For preparing a therapeutic formulation, the carrier substances, which are very frequently lipids, are taken as such or dissolved in a physiologically acceptable solvent or a water-miscible solubilizing agent, combined with a polar solution, and made to form carriers.

It is advantageous to use polar solutions containing edge active substances; the latter can also be used with lipids or be contained in a lipid solution.

Carrier formation is preferably initiated by stirring in, by means of evaporation from a reverse phase, by means of an injection or a dialysis procedure, through mechanical agitation, such as shaking, stirring, homogenization,

ultrasonication, friction, shear, freezing-and-thawing, by means of high-and low-pressure filtration, or any other use of energy.

It may be advantageous to incorporate agents only after carrier formation.

If transfersomes are prepared by means of filtration, materials with a pore size of 0.1-0.8 micrometers, very frequently of 0.15-0.3 micrometers, and particularly preferred of 0.22 micrometers are preferably used; several filters can also be used in combination or in a row.

In the case that transfersomes are made by means of ultrasonication, energy densities in the order of 10-50 kW/litre/minute are preferably used; in stirring or rotary machines 1,000 through to 5,000 revolutions per minute are typically used. If high pressure homogenizers are used, pressures in the order of 300-900 Bar normally ensure sufficient transfersome homogeneity and quality after a single passage; in the latter case even suspensions with 20-30 % lipids can be processed without any difficulty.

It is often sensible to prepare transfersomes only shortly before an application from a concentrate or lyophylisate.

Cryopreservatives, such as oligosaccharides, can facilitate the formation of transfersomes from a lyophylisate.

Standard agent, supporting, or additional substances, in particular the stabilizing, protective, gel-forming, appearance-affecting substances and markers can also be used as described in this application.

The following examples illustrate this invention without

implying any limits to its general use. Temperatures are given in degree Celsius, carrier sizes in nanometers, and other quantities in common SI units.

Example 237:

Composition:

120 mg	phosphatidylcholine from soy-bean (purity > 95 %)
20 mg	sodium-cholate p.a. (L/D = 3,2)
150 µl	ethanol (96 %)
1.45 ml	Actrapid HM 100 (recombinant human insulin 100 I.U./ml)

Preparation:

This preparation is produced as described in example 166, with only minor modifications. The main difference is that the lipid/insulin mixture is hand-filtered through a 0.22 µm polycarbonate filter (Sartorius) using a 1 ml injection already few minutes after mixture preparation. The final volume of the suspension is 1.2 ml; the nominal lipid/cholate ratio is 2.8/1, in lipid membranes approx. 2.4/1. The final concentration of insulin is approx. 83 I.U./ml; the vesicle radius one day after preparation is 94 nm on the average; one week later, 170 nm.

Application:

One and half hours after the beginning of the experiment, 240 µl of a sterile suspension of insulin containing transfersomes (with 20 I.U.) were taken. These were applied and uniformly smeared at a dose of approx. 0.7 mg lipid/cm^2 over the inner side of the right forearm of a male test person starved for 18

hours prior to experiment. 5 minutes later the skin surface is macroscopically dry. Another 45 minutes later no traces of application are visible anymore.

Activity:

At irregular intervals of between 15 and 40 minutes, blood samples are drawn from a soft i.v. catheter placed in the left forearm. The determination of the blood glucose level is performed as described in example 166.

The course in time of the transfersome mediated hypoglycemia is represented in figure 18. The blood glucose level decreases approx. 1.5 hours after drug application by some 10 mg/ml; this artificial hypoglycemia lasts for 4 hours at least and thus attains 70-80 % of the value which can be achieved by a subcutaneous application of a comparable amount of the drug Actrapid. The results of control experiments in which the insulin containing transfersomes are injected subcutaneously are shown as crosses in this figure. The total effect in the latter case is similar to that induced by the free drug injected s.c.

Example 238:

Composition:

216 mg	phosphatidylcholine from soy-bean (487 µl of a 50 % solution in absolute ethanol)
27 mg	phosphatidylglycerol from egg (98 %)
29.45 mg	oleic acid, puriss.
3 ml	Actrapid HM 100 (recombinant human insulin 100 I.U./ml)
40 µl	1 N NaOH
20 µl	1 N NaCl

Preparation:

Lipids are mixed until solution is homogeneously clear. After the addition of an actrapid solution, of alkali and salt solution, an optically opalescent suspension is formed. Filtering of this suspension through a polycarbonate filter with a pore diameter of 0.2 μm yields a much less opalescent suspension which consists of vesicles (transfersomes) with a mean diameter of 320 nm.

Application:

Starting glucose concentration in the blood of a test person (70 kg, 37 years, normoglycemic, starved for 24 hours) is measured over a period of 90 minutes for reference. Subsequently, the above-mentioned transfersome suspension with a nominal concentration of 85 I.U. insulin/ml, which has been aged for 12 hours at 4°C, is applied on the right forearm skin (approx. 330 μl over an area of approx. 15 cm^2); this corresponds to a total applied dose of 28 I.U.

Activity:

Blood specimens are collected through a heparinized, permanent, soft catheter placed in a vein in the left forearm; 0.5 ml of each sample are sedimented and immediately frozen for further use. The remaining volume is used for the in situ determination of the blood glucose concentration by an enzymatic method. The measured glucose concentration decreases by approx. 8 mg/dl after approx. 2.5 hours and remains diminished for more than 4.4 hours. This corresponds to 75 % of the maximally achievable effect, as concluded from control experiments performed by injecting insulin s.c. The pharmacokinetics of this experimental series is represented in

figure 19.

Figure 20 gives the results of three typical experiments with insulin. They illustrate the results obtained by one percutaneous and two s.c. drug applications.

Example 239:

Composition:

143 mg	phosphatidylcholine from soy-bean
18 mg	phosphatidylglycerol from egg (98 %)
19.6 mg	oleic acid, puriss.
2 ml	Actrapid HM 100 (200 I.U.)
25 µl	1 N NaOH

Preparation:

Lipids are weighed into a glass vial and mixed with a standard insulin solution. The resulting opaque suspension is ultrasonicated directly, using a titanium probe-tip (approx. 5 W, 3x5 seconds at 22°C in 60 seconds intervals). The resulting, optically clear but still opalescent suspension contains vesicles with a mean radius of 114 ± 17 nm.

Application and Activity:

The results of this test series are within the limits of experimental error identical to those obtained in example 238.

Example 240:

Composition:

143 mg	phosphatidylcholine from soy-bean

18 mg	phosphatidylglycerol from egg (98 %)
20.5 mg	sodium oleate
2 ml	Actrapid HM 100 (200 I.U.)

Preparation:

The lipids are dissolved in a glass vial in 0.15 ml abs. ethanol and then combined with a standard insulin solution. Further procedure is as described in example 239.

Application and Activity:

Over an area of approx. 5 cm^2 on the forearm skin of a test person a piece of fine-mesh synthetic cloth is fixed. This is then covered with 350 μl of an insulin containing transfersome suspension and left uncovered to dry.

The resulting decrease of the blood glucose level after 4 hours amounts to 7.8 mg/dl and after 6 hours to 8.5 mg/dl. It is thus comparable to the result obtained in experiment no. 238.

Example 241:

The procedure is at first as described in example 238 except that no salt solution is added to the sample suspension; the opaque crude transfersome suspension is divided into two parts. One of these consisting of 50 % of the total volume is passed through a sterile filter; the other half is ultrasonicated for 15 seconds at room temperature at a power of approx. 5 W. The mean diameter of carriers in both halves is similar, 300 nm or 240 nm, respectively.

Example 242:

The procedure is as described in examples 238 and 240. Transfersomes, however, are filtered one, two and three times in a row. The mean vesicle diameter in the resulting three samples are 300, 240, and 200 nm, resp..

The transfersomes of examples 241 and 242 yield similar hypoglycemic results in biological tests as those of example 238.

Example 243:

Composition:

144,9;152 mg	phosphatidylcholine from soy-bean
24.8;17.6 mg	desoxycholate, Na-salt
1.45;1.55 ml	Actrapid HM 100 (145 I.U.)
0.16 ml	ethanol, absolute

Preparation:

Lipids are weighed into glass vials, dissolved with ethanol and mixed with an insulin solution. The resulting opaque suspension is aged over night and subsequently filtered through a 0.22 micrometer filter at t=12 hours. The nominal insulin concentration is 83 or 84 I.U; the mean vesicle radius in both cases is 112 nm.

Application and Activity:

General experimental conditions are as described in examples 237-239. Transfersome suspensions (0.36 ml, corresponds to 30 I.U.) are applied onto the inner side of a forearm skin in both cases; the blood samples are taken from a soft catheter placed in a vein in the other forearm.

The results of these two experiments are given in figure 21. They show that preparations with a relatively high surfactant concentration (Sample 1, L/S=3/1) can cause a hardly significant decrease in the blood glucose level; transfersomes close to their optimum, however, with a surfactant concentration lower by approx. 30 % (L/S=4.5/1), cause a very pronounced 'hypoglycemia' which lasts for many hours.

This is another proof that the transfersomes tend to transport drugs through intact skin according to a completely new principle of action which is dissimilar to that of classical pharmaceutical formulations.

This example, in addition to example 236, furthermore, suggests the following conclusion: for the systems investigated, also surfactant concentrations can be used which are remote from the transfersomal optimum (without the carrier activity being lost completely); notwithstanding this, particularly advantageous results are obtained when the surfactant concentration has been determined and chosen to be in a range which ensures maximum carrier elasticity and thus permeation capability of the transfersomes in combination with sufficiently high carrier stability to dissolution, bursting, agent loss, etc.

Claims:

1. Preparation for the application of agents in the form of minute droplets of fluid, in particular with a membrane-like coating consisting of one or several layers of amphiphilic molecules or of one amphiphilic carrier substance, for transporting agents into and through natural barriers and constrictions such as skin and similar materials, characterized by the fact that each preparation contains an edge active substance at a concentration which amounts up to 99 mol-% of the concentration of this substance required to solubilize the droplet.

2. Preparation according to claim 1, wherein the concentration of edge active substance amounts to at least 0.1 mol-%, in particular between 1 and 80 mol-%, preferably between 10 and 60 mol-%, and particularly preferred between 20 and 50 mol-% of the solubilization-inducing concentration of edge active substances, whereby the edge activity of a droplet unit is preferably close to approx. 10 Piconewton or less.

3. Preparation according to claims 1 or 2, characterized by the fact that the preparation contains an amount of an amphiphilic substance as a carrier or as a basis for the membrane-like envelope of the droplet forming hydrophilic fluid, the agent being contained in the carrier substance, in the shell, and/or in the droplet material itself.

4. Preparation as claimed in claim 3, wherein said amphiphilic substance is a lipid-like material and said edge active substance is preferably a surfactant.

5. Preparation as claimed in one of claims 1 through 4, wherein the content of said amphiphilic substance for the applications on human or animal skin amounts to 0.01 through 30 weight-% of the preparation mass, preferably between 0.1 and 15 weight-% and particularly preferred between 5 and 10 weight-%.

6. Preparation as claimed in one of claims 1 through 4, wherein the content of the amphiphilic substance in the formulation for application on plants is 0.000001 through 10 weight-%, preferably between 0.001 and 1 weight-% and particularly preferred between 0.01 and 0.1 weight-%.

7. Preparation as claimed in any one of the preceding claims, wherein an agent is an adrenocorticostatic, a ß-adrenolytic, an androgen or antiandrogen, antiparasitic, anabolic, anaesthetic or analgesic, analeptic, antiallergic, antiarrhythmic, antiarterosclerotic, antiasthmatic and/or bronchospasmolytic, antibiotic, antidrepressant and/or antipsychotic, antidiabetic, an antidote, antiemetic, antiepileptic, antifibrinolytic, anticonvulsive, an anticholinergic, an enzyme, coenzyme or a corresponding inhibitor, an antihistaminic, antihypertonic, a biological inhibitor of drug activity, an antihypotonic, anticoagulant, antimycotic, antimyasthenic, an agent against Morbus Parkinson, an antiphlogistic, antipyretic, antirheumatic, antiseptic, a respiratory analeptic or a respiratory stimulant, a broncholytic, cardiotonic, chemotherapeutic, a coronary dilatator, a cytostatic, a diuretic, a ganglium-blocker, a glucocorticoid, an antiflew agent, a haemostatic, hypnotic, an immunoglobuline or its fragment or any other immunologically active substance, a bioactive carbohydrate (derivative), a contraceptive, an anti-migraine agent, a mineralcorticoid, a morphine-

antagonist, a muscle relaxant, a narcotic, a neuraltherapeutic, a nucleotide, a neuroleptic, a neurotransmitter or some of its antagonists, a peptide(derivative), an opthalmic, (para)-sympaticomimetic or (para)sympathicolytic, a protein(derivative), a psoriasis/neurodermitis drug, a mydriatic, a psychostimulant, rhinologic, any sleep-inducing agent or its antagonist, a sedating agent, a spasmolytic, tuberlostatic, urologic, a vasoconstrictor or vasodilatator, a virustatic or any wound-healing substance, or several such agents.

8. Preparation as claimed in one of claims 1 through 6, wherein said agent is a growth modulating substance for living organisms.

9. Preparation as claimed in one of claims 1 through 6, wherein said agent exerts some biocidal activity and particularly is an insecticide, a pesticide, a herbicide or a fungicide.

10. Preparation as claimed in one of claims 1 through 6, wherein an agent is an attractant, in particular from the class of pheromones.

11. A method for manufacturing preparations for the application of agents in the form of minute droplets of a fluid, in particular in a membrane-like 'envelope' consisting of one or several layers of amphiphilic molecules, or supplemented with an amphiphilic carrier substance, in particular for the transport of agents in and through natural barriers and constrictions, such as skin and the like, characterized by the fact that the concentration of an edge active substance required for the solubilization of a carrier entity is determined and

then an amount of the edge active substance which is close to the former concentration but still guarantees a sufficient carrier stability and permeation capability is used for the preparation.

12. Method as claimed in claim 11, wherein the stability and the permeation capacity of the fluid 'droplet' are determined by means of filtration, if required under pressure, through a fine-pore filter or by means of any other controlled mechanical fragmentation.

13. Method as claimed in claims 11 or 12, wherein the content of said edge active substance is between 0.1 and 99 mol-%, and in particular between 1 and 80 mol-%, preferably between 10 and 60 mol-% and most preferred between 20 and 50 mol-% of the concentration at which solubilization of the carrier is achieved.

14. Method as claimed in one of claims 11 through 13, wherein said mixture of substances required for the formation of a preparation is subjected to filtration, ultrasonication, stirring, agitating or any other mechanical fragmentation.

15. Preparation as claimed in one of claims 1 through 10, wherein said preparation for non-invasive application contains at least one antidiabetic agent, in particular insulin.

16. Preparation as claimed in claim 15, characterized by the fact that it contains a physiologically compatible polar or non-polar lipid as an amphiphilic carrier substance, the carrier membrane preferably having a double layer structure.

17. Preparation as claimed in claim 16, wherein the amphiphilic substance is a lipid or a lipoid from any biological source or a corresponding synthetic lipid, or else comprises a modification of such lipids, a glyceride, in particular glycerophospholipid, isoprenoidlipid, sphingolipid, steroid, sterin or sterol, a sulfur- or carbohydrate-containing lipid, or else any other lipid which forms stable double layers, preferably a half-protonated fluid fatty acid, and preferably a phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, a phosphatidic acid, a phosphatidylserin, a sphingomyelin or sphingophospholipid, glycosphingolipid (e.g. cerebroside, ceramidepolyhexoside, sulfatide, sphingoplasmalogene), a ganglioside or any other glycolipid or a synthetic lipid, preferably a dioleoyl-, dilinoleyl-, dilinolenyl-, dilinolenoyl-, diarachidoyl-, dimyristoyl-, dipalmitoyl, distearoyl, phospholipid or corresponding sphingosinderivative, a glycolipid or any other diacyl- or dialkyl-lipid.

18. Preparation as claimed in one of claims 15 through 17, containing several edge active substances.

19. Preparation as claimed in one of claims 15 through 18, wherein said edge active substance is a nonionic, a zwitterionic, an anionic or a cationic surfactant, in particular a long-chain fatty acid or a long-chain fatty alcohol, an alkyl-trimethyl-ammonium-salt, alkylsulfate-salt, cholate-, deoxycholate-, glycodeoxycholate-, taurodeoxycholate-salt, dodecyl- dimethyl-aminoxide, decanoyl- or dodecanoyl-N- methylglucamide (MEGA 10, MEGA 12), N-dodecyl-N,N- dimethylglycine, 3-

(hexadecyldimethylammonio)-propane-sulfonate, N-hexadecyl-sulfobetaine, nonaethylene-glycol-octylphenylether, nonaethylene-dodecylether, octaethyleneglycol-isotridecylether, octaethylene-dodecylether, polyethylene glycol-20-sorbitane-monolaurate (Tween 20), polyethylene glycol-20-sorbitane-monooleate (Tween 80), polyhydroxyethylene-cetylstearyl ether (Cetomacrogo, Cremophor O, Eumulgin, C 1000) polyhydroxyethylene-4-laurylether (Brij 30), polyhydroxyethylene-23-laurylether (Brij 35), polyhydroxyethylene-8-stearate (Myrj 45, Cremophor AP), polyhydroxyethylene-40-stearate (Myrj 52), polyhydroxyethylene-100-stearate (Myrj 59), polyethoxylated castor oil 40 (Cremophor EL), polyethoxylated hydrated castor oil, sorbitane-monolaurate (Arlacel 20, Span 20), particularly preferred decanoyl- or dodecanoyl-N-methylglucamide, lauryl- or oleoylsulfate-salts, sodiumdeoxycholate, sodiumglycodeoxycholate, sodiumoleate, sodiumelaidate, sodiumlinoleate, sodiumlaurate, nonaethylene-dodecyl-ether, polyethylene glycol-20-sorbitane-monooleate (Tween 80), polyhydroxyethylene-23-laurylether (Brij 35), polyhydroxyethylene-40-stearate (Myrj 52) and/or sorbitane-monolaurate (Arlacel 20, Span 20) and lysophospholipid, such as n-octadecylen(=oleoyl)-glycero-phosphatidic acid, -phosphorylglycerol, or -phosphoryl-serine, n-dilauryl-glycero-phosphatidic acid, -phospho-ryl glycerol, or -phosphorylserine, n-tetradecyl-glycero-phosphatidic acid, -phosphorylglycerol, or -phosphorylserine and corresponding palmitoeloyl-, elaidoyl-, vaccenyl-lysophospholipids.

20. Preparation as claimed in one of claims 15 through 19, characterized by the fact that it contains 1 through 500 I.U. insulin/ml as agent, preferably between 20 and 100

I.U. insulin/ml and the concentration of the carrier substance in the preparation is in the range of 0.1 through 20 weight-%, in particular between 0.5 and 15 weight-%, particularly preferred between 2.5 and 10 weight-%.

21. Preparation as claimed in one of claims 15 through 20, characterized by the fact that a phosphatidylcholine and/or a phosphatidylglycol is used as an amphiphilic substance, and that a lysophosphatidic acid or lysophosphoglycerol, a deoxycholate-, glycodeoxycholate- or cholate salt, a laurate, myristate, oleate, palmitoleate, or a corresponding phosphate- or sulfate-salt, and/or a Tween- or a Myrj-surfactant is used as an edge active substance, recombinant human insulin being the preferred agent.

22. Preparation as claimed in one of claims 15 through 21, wherein the radius of said vesicular droplets in a preparation is between approx. 50 and approx. 200 nm, preferably between approx. 100 and 180 nm.

23. A method for the preparation of a formulation for the non-invasive application of antidiabetic agents, wherein said liposome-like droplets are formed from at least one amphiphilic substance, at least one hydrophilic fluid, at least one edge active substance, and at least one antidiabetic agent which together form the preparation.

24. Method as claimed in claim 23, wherein the edge active substance and the amphiphilic substance, and the hydrophilic substance and the agent are separately mixed together and, if required, dissolved in a solution, the resulting mixtures or solutions then being combined

as one mixture to induce the formation of carrier particles, particularly by action of mechanical energy.

25. Method as claimed in claims 23 or 24, wherein said ampiphilic substance is either used as such or dissolved in a physiologically compatible solvent which is very frequently miscible with hydrophilic fluids, in particular water, or in a solvation mediating agent together with a polar solution.

26. Method as claimed in claim 25, wherein the polar solution contains at least one edge active substance.

27. Method as claimed in one of claims 23 through 26, characterized by the fact that the formation of droplets is induced by substance addition into a fluid phase, evaporation from a reverse phase, using an injection- or dialysis procedure, with the aid of mechanical stress such as shaking, stirring, homogenizing, ultrasonication, shear, freezing and thawing, or high- or low-pressure filtration.

28. Method as claimed in claim 27, characterized by the fact that the formation of droplets is caused by filtration the filtering material having pore diameters of 0.1 through 0.8 μm, in particular with 0.15 through 0.3 μm, especially preferred 0.22 μm, several filters being sometimes used in a sequence.

29. Method as claimed in one of claims 23 through 28, wherein inclusion of said agents occurs at least partly after the droplet formation.

30. Method as claimed in one of claims 23 through 29, wherein liposome-like droplets are prepared just before

their application from a suitable concentrate or a lyophylisate.

Abstract

The invention relates to a preparation for the application of agents in the form of minuscule droplets of fluid, in particular provided with membrane-like structures consisting of one or several layers of amphiphilic molecules, or an amphiphilic carrier substance, in particular for transporting the agent into and through natural barriers such as skin and similar materials. The preparation contains a concentration of edge active substances which amounts to up to 99 mol-% of the agent concentration which is required for the induction of droplet solubilization. Such preparations are suitable, for example, for the non-invasive applications of antidiabetics, in particular of insulin. The invention, moreover, relates to the methods for the preparation of such formulations.

FIGURE 1

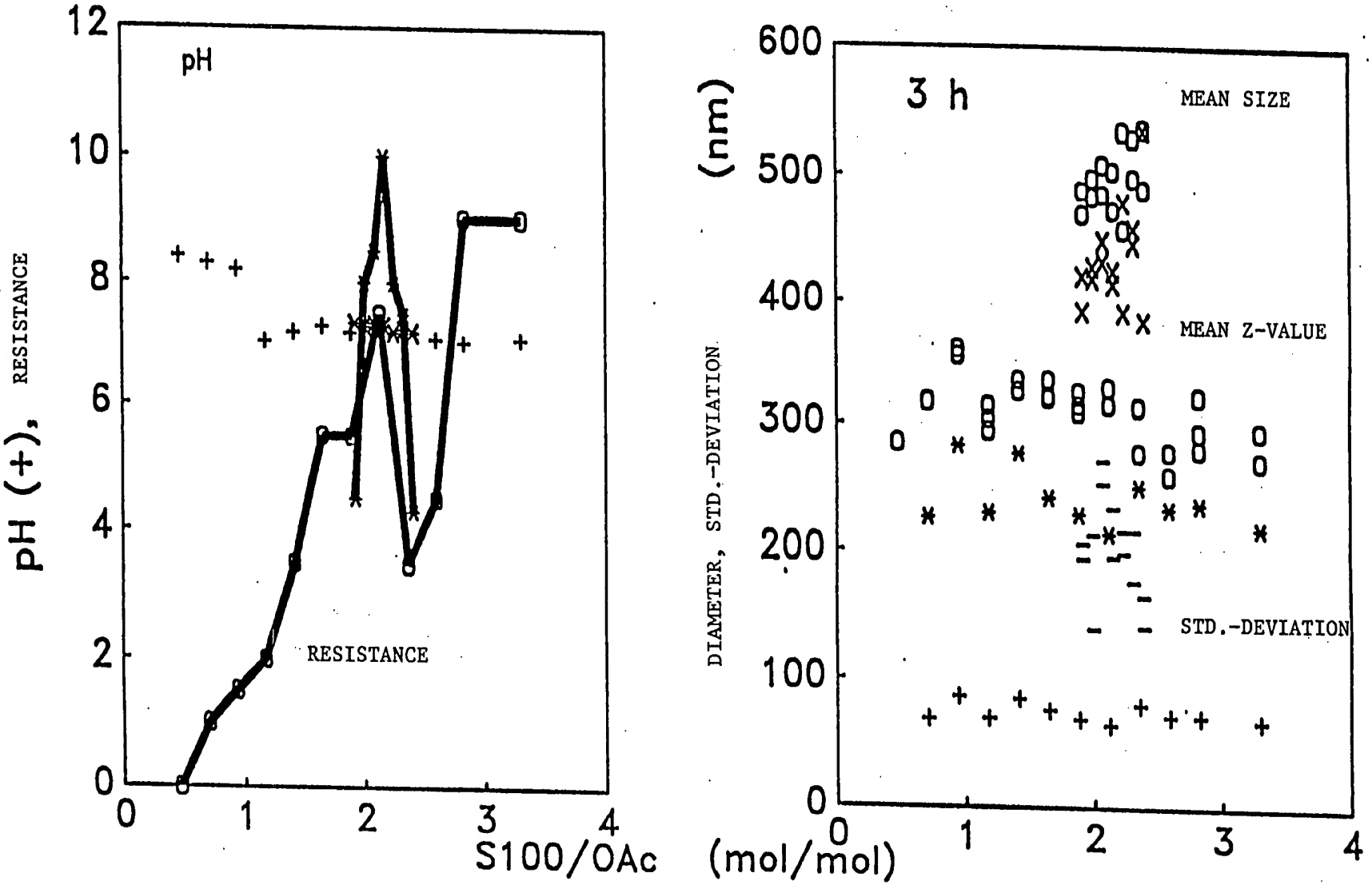

pH
RESISTANCE
pH (+), RESISTANCE
S100/OAc
3 h
MEAN SIZE
MEAN Z-VALUE
STD.-DEVIATION
DIAMETER, STD.-DEVIATION (nm)
(mol/mol)

FIGURE 2

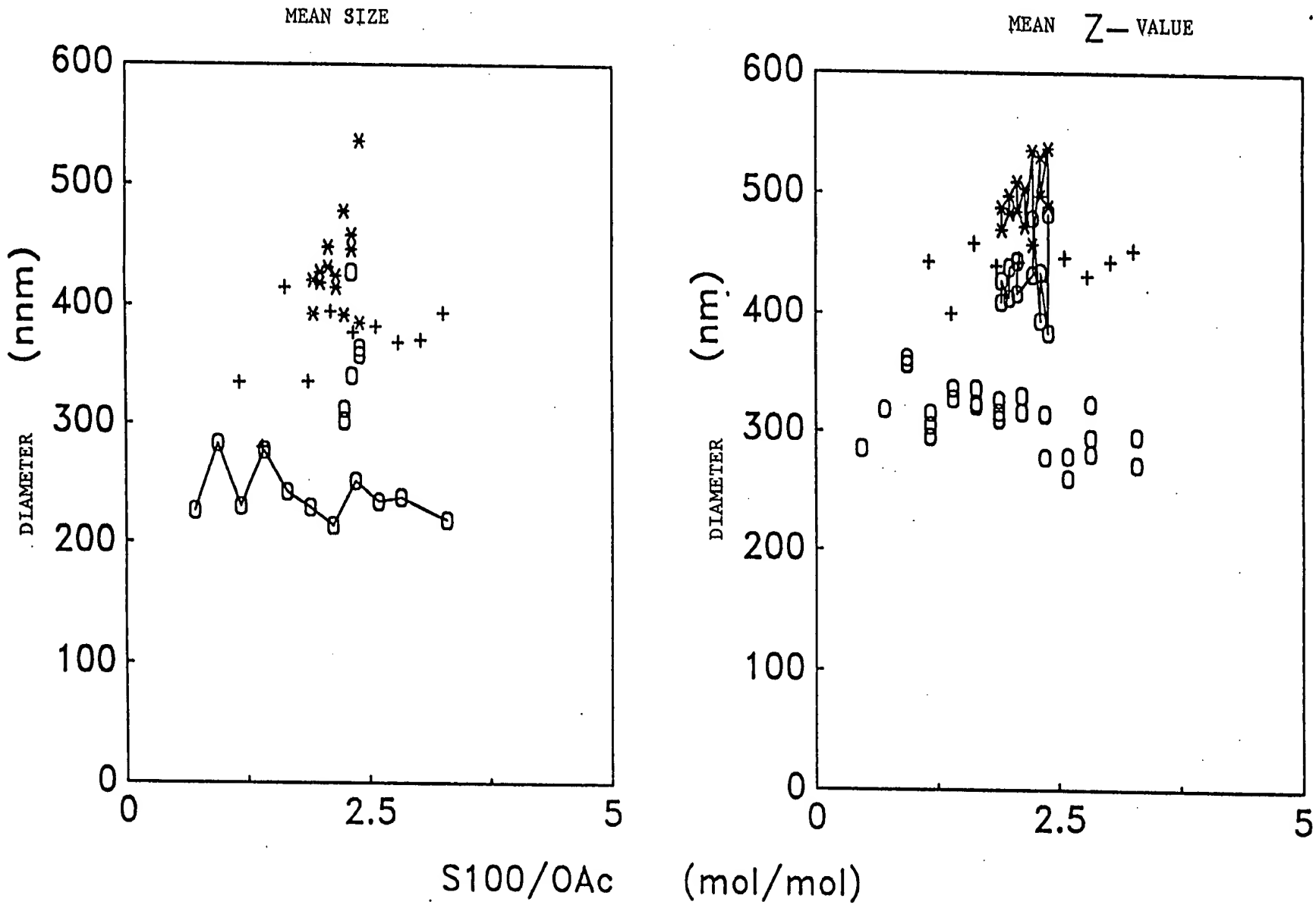
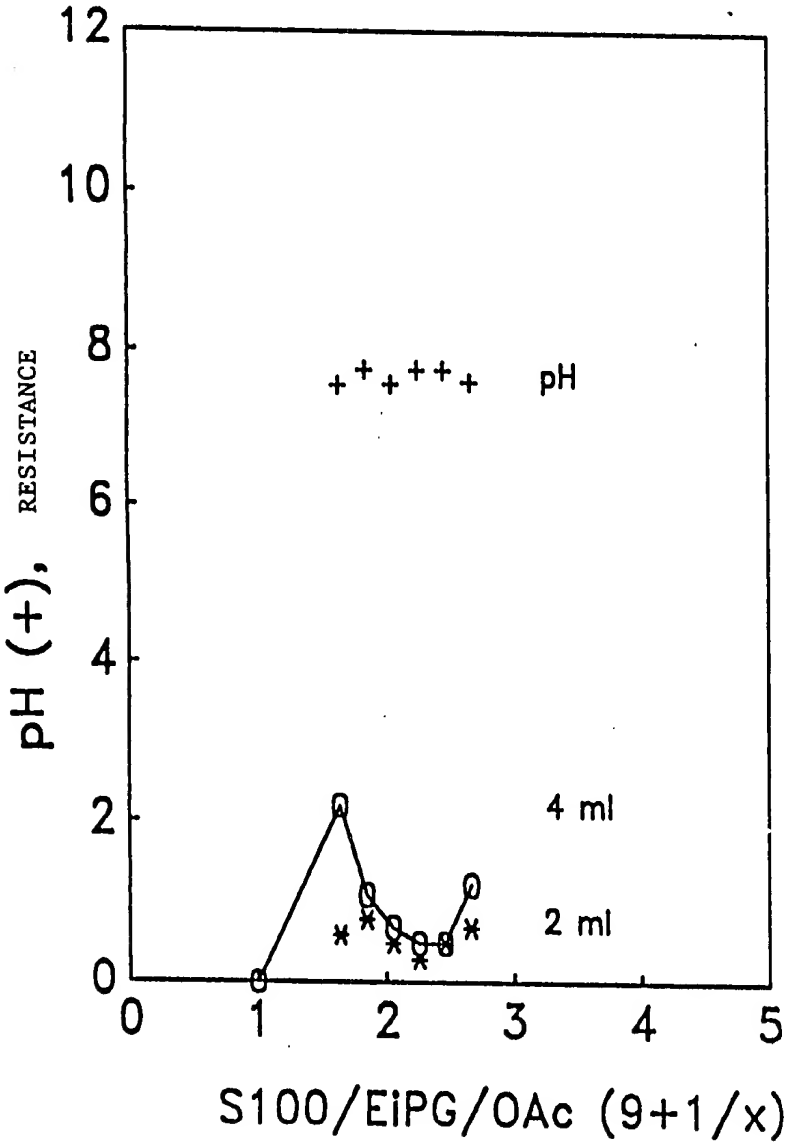

MEAN SIZE
600
500
400
300
200
100
0
(nnm)
DIAMETER
0
2.5
5
S100/OAc (mol/mol)
MEAN Z— VALUE
600
500
400
300
200
100
0
(nm)
DIAMETER
0
2.5
5

FIGURE 3


12
10
8
6
4
2
0
pH (+), RESISTANCE
pH
4 ml
2 ml
0
1
2
3
4
5
S100/EiPG/OAc (9+1/x) (mol/mol)

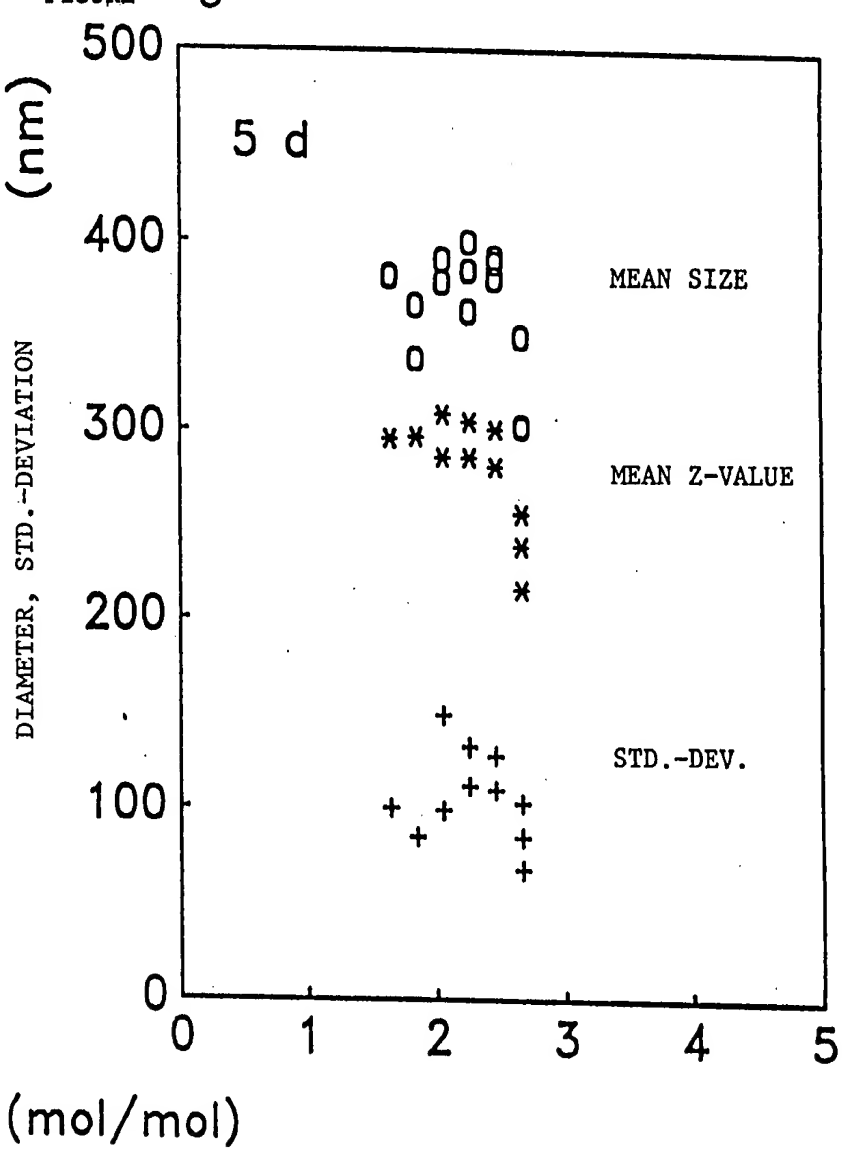

500
400
300
200
100
0
DIAMETER, STD.-DEVIATION (nm)
5 d
MEAN SIZE
MEAN Z-VALUE
STD.-DEV.
0
1
2
3
4
5

FIGURE 4

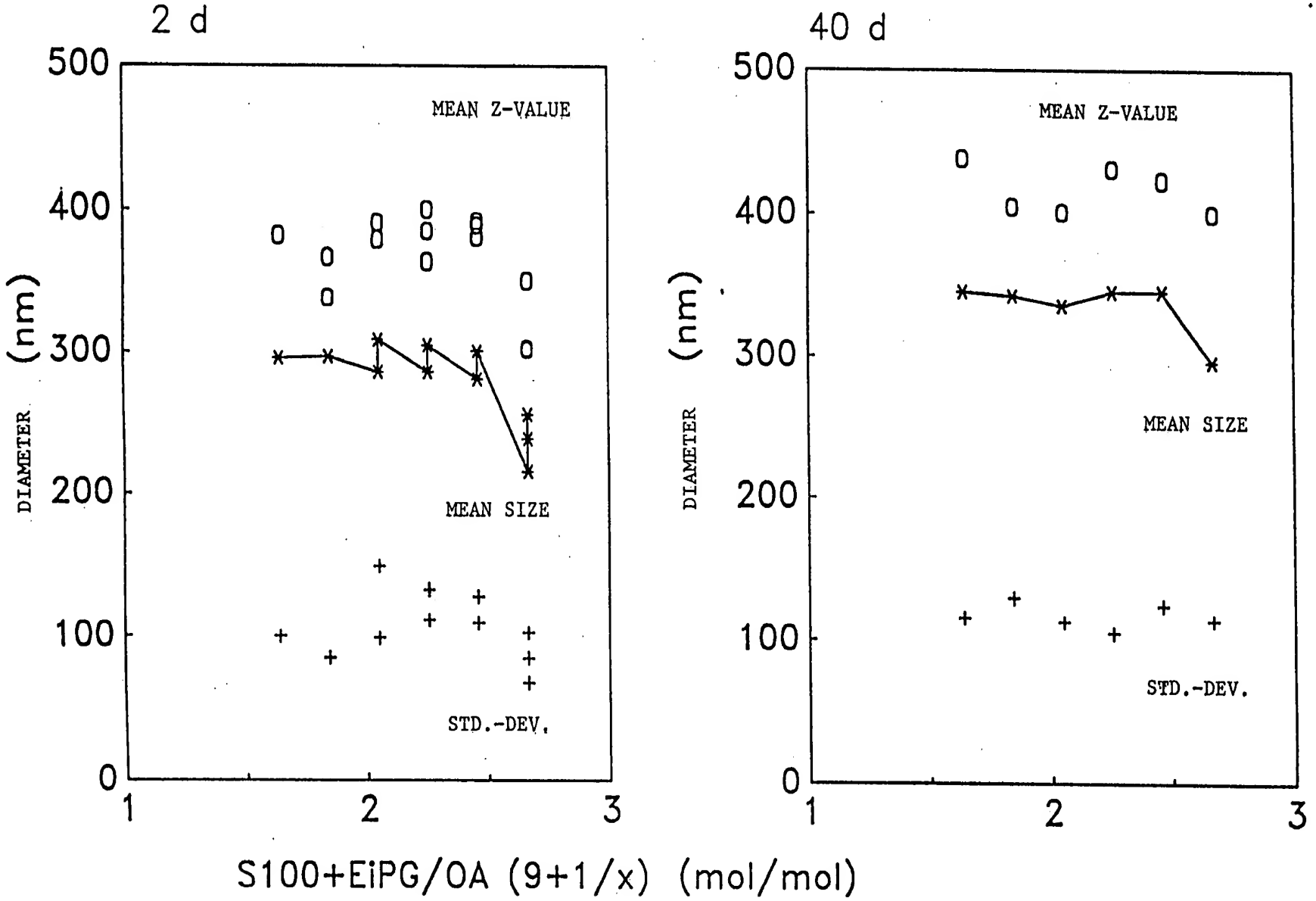

2 d
40 d
500
400
300
200
100
0
DIAMETER (nm)
MEAN Z-VALUE
MEAN SIZE
STD.-DEV.
1
2
3
S100+EiPG/OA (9+1/x) (mol/mol)

FIGURE 5

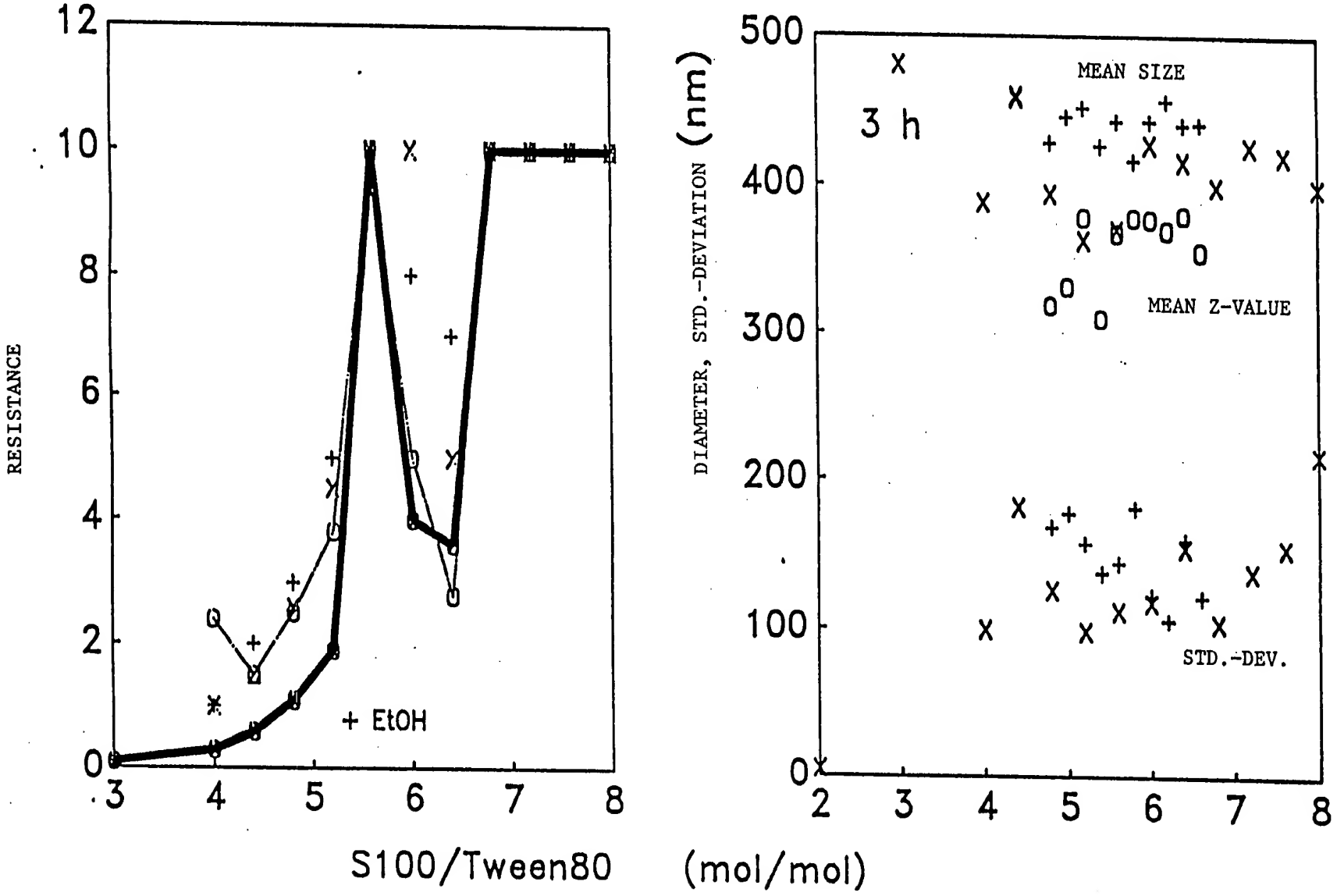

RESISTANCE
12
10
8
6
4
2
0
3
4
5
6
7
8
+ EtOH
S100/Tween80
DIAMETER, STD.-DEVIATION (nm)
500
400
300
200
100
0
2
3
4
5
6
7
8
3 h
MEAN SIZE
MEAN Z-VALUE
STD.-DEV.
(mol/mol)

FIGURE 6

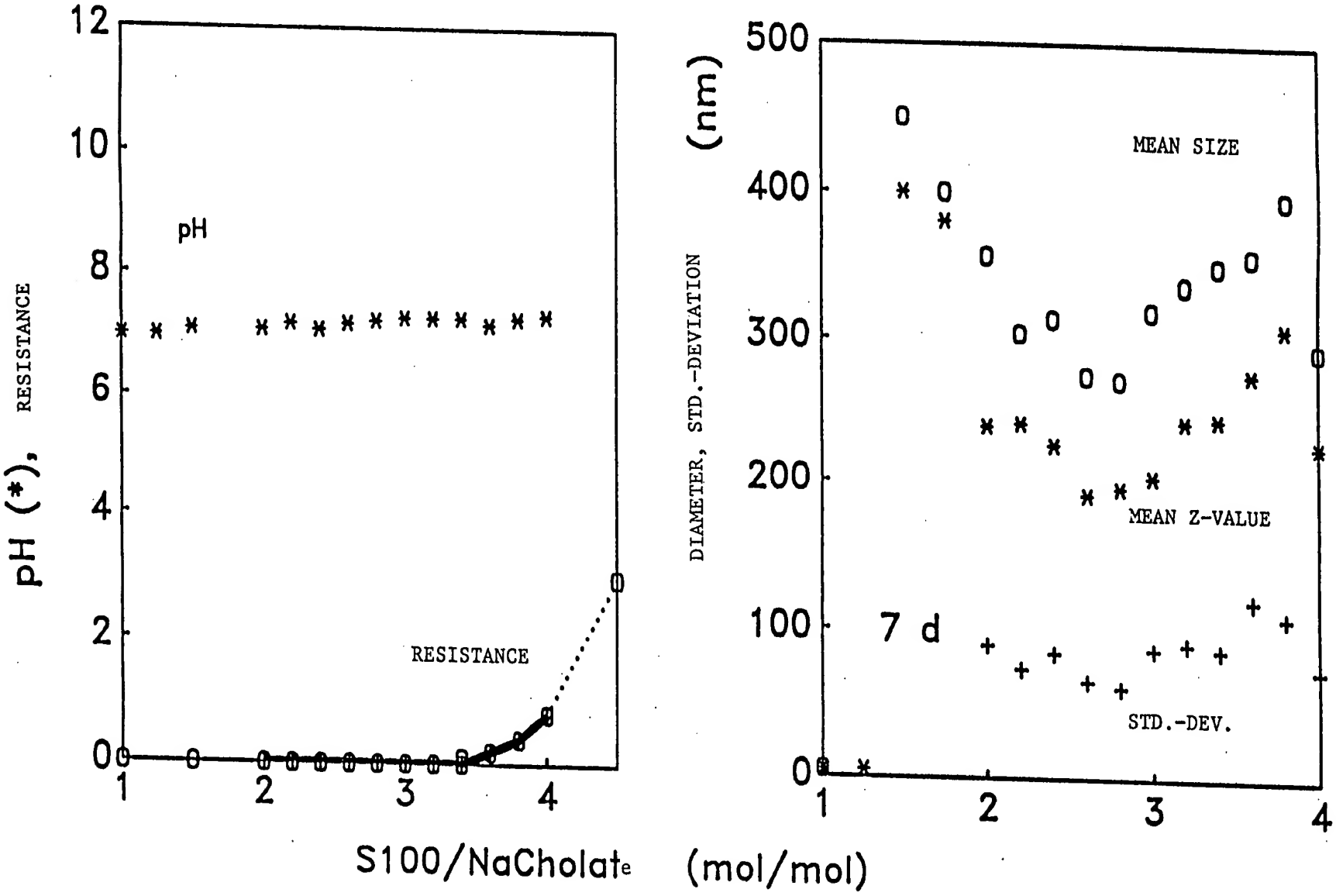

pH (*), RESISTANCE
12
10
8
6
4
2
0
pH
RESISTANCE
1
2
3
4
S100/NaCholate (mol/mol)
DIAMETER, STD.-DEVIATION (nm)
500
400
300
200
100
0
MEAN SIZE
MEAN Z-VALUE
7 d
STD.-DEV.
1
2
3
4

FIGURE 7

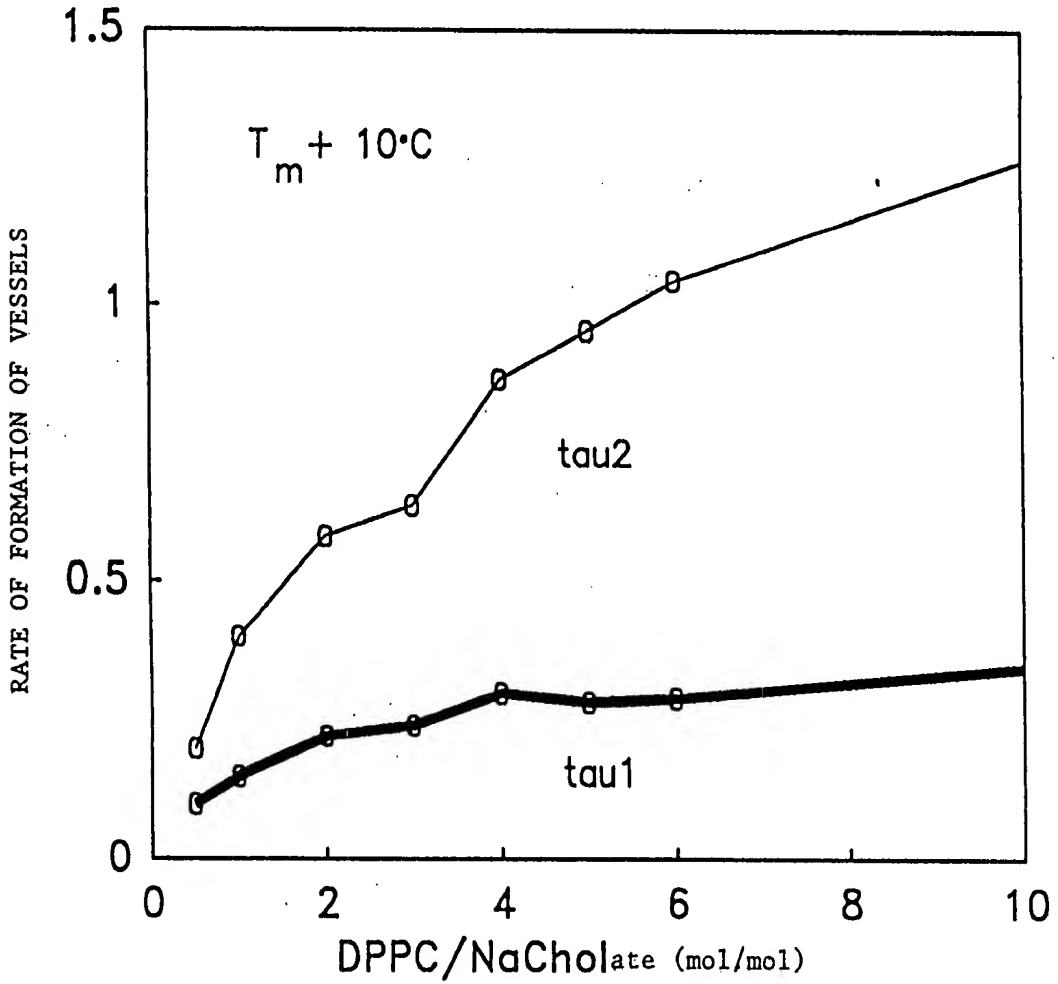

1.5
1
0.5
0
T_m + 10°C
tau2
tau1
RATE OF FORMATION OF VESSELS
0
2
4
6
8
10
DPPC/NaCholate (mol/mol)

FIGURE 8

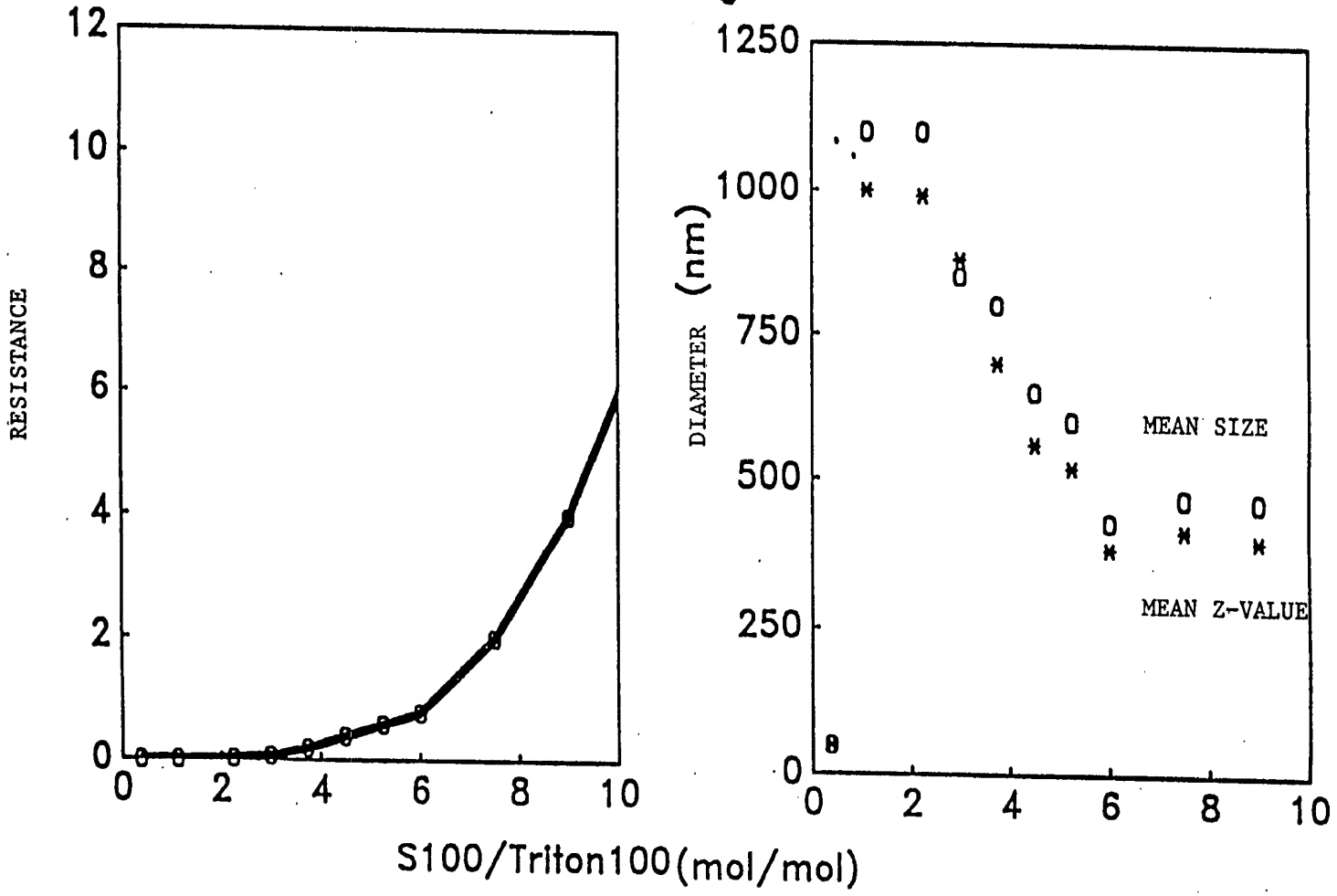

RESISTANCE
12
10
8
6
4
2
0
0
2
4
6
8
10
DIAMETER (nm)
1250
1000
750
500
250
0
MEAN SIZE
MEAN Z-VALUE
S100/Triton100(mol/mol)

FIGURE 9

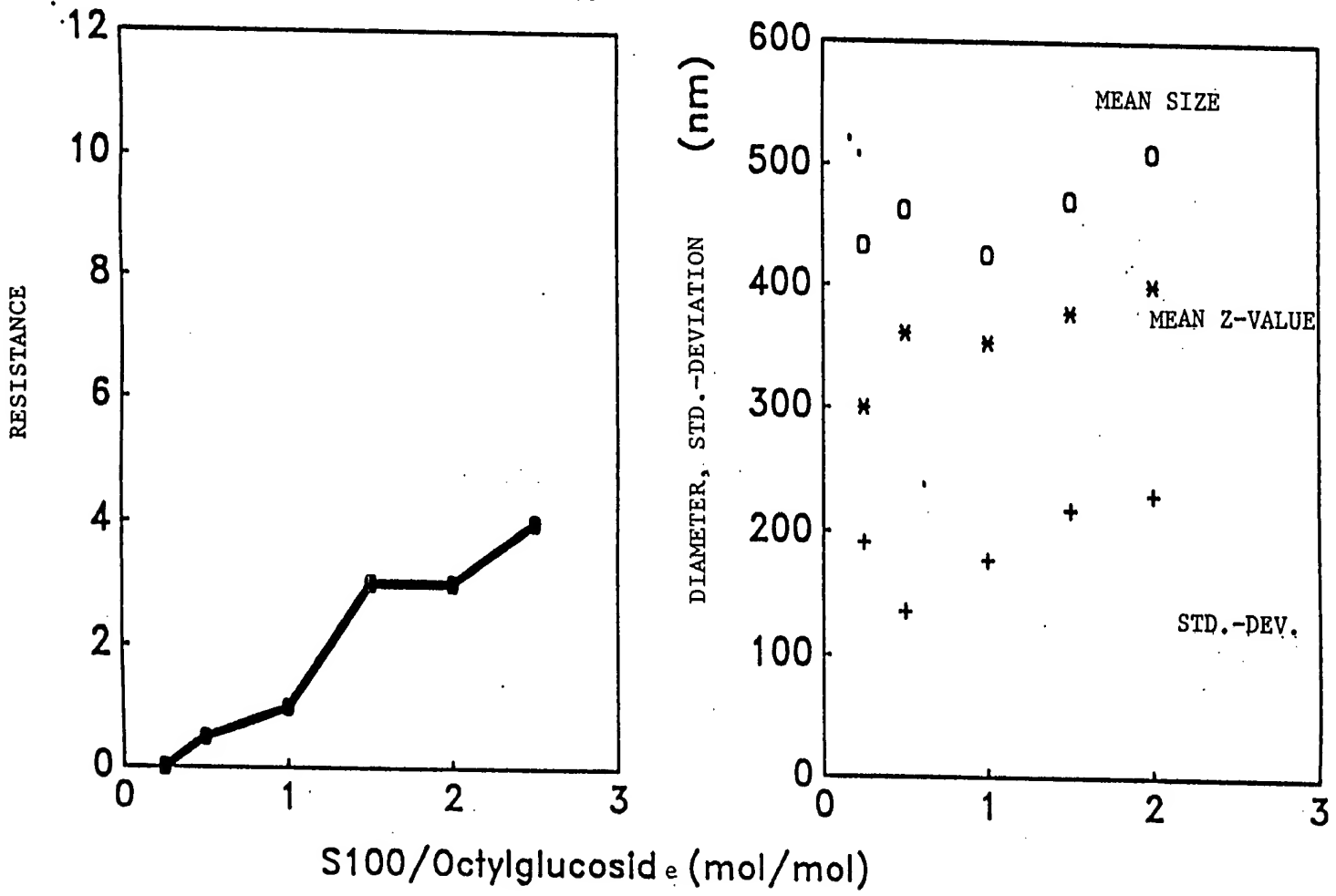
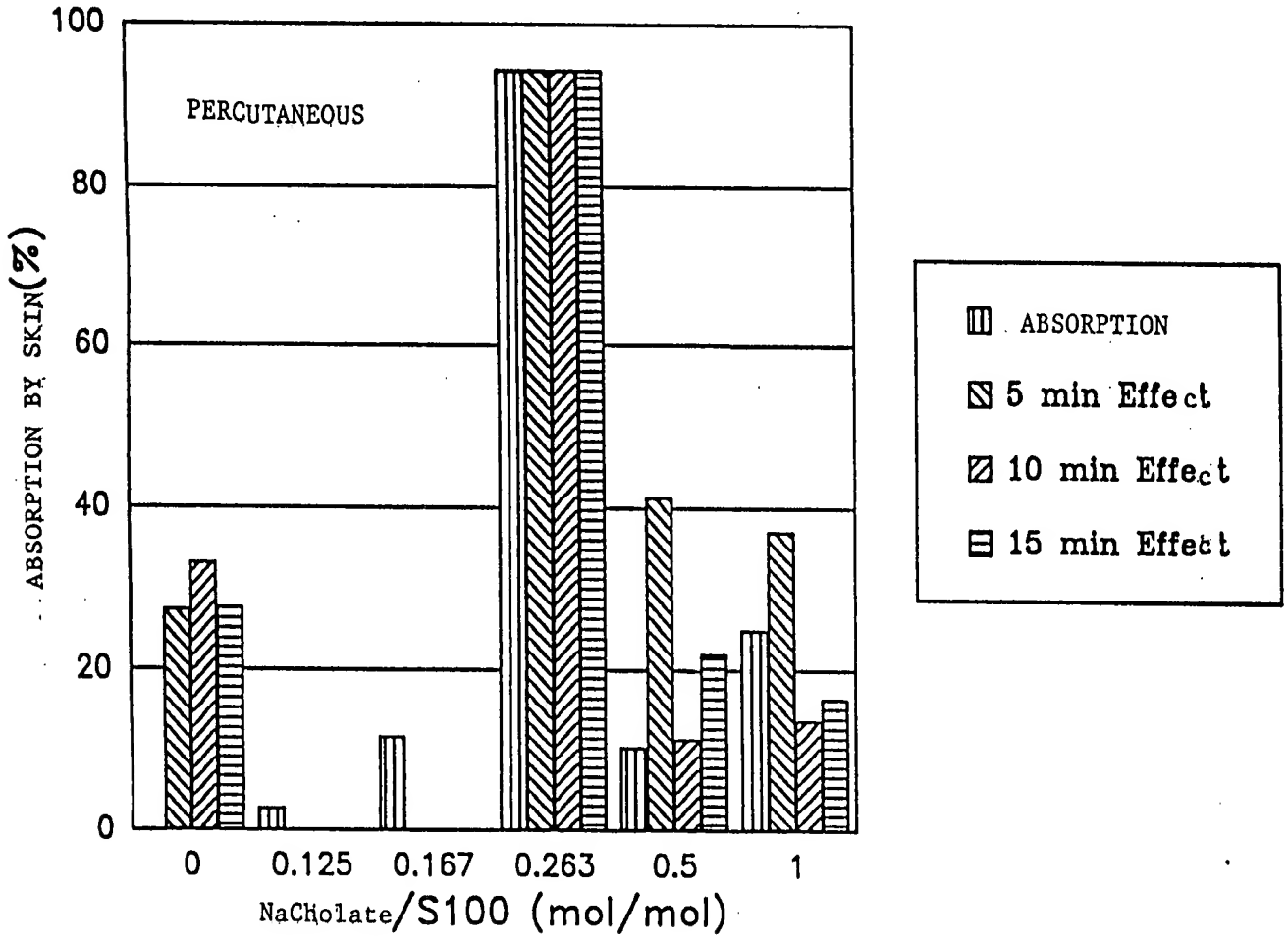

RESISTANCE
12
10
8
6
4
2
0
0
1
2
3
DIAMETER, STD.-DEVIATION (nm)
600
500
400
300
200
100
0
MEAN SIZE
MEAN Z-VALUE
STD.-DEV.
S100/Octylglucoside (mol/mol)

FIGURE 10


100
80
60
40
20
0
PERCUTANEOUS
ABSORPTION BY SKIN(%)
0
0.125
0.167
0.263
0.5
1
NaCholate/S100 (mol/mol)
ABSORPTION
5 min Effect
10 min Effect
15 min Effect

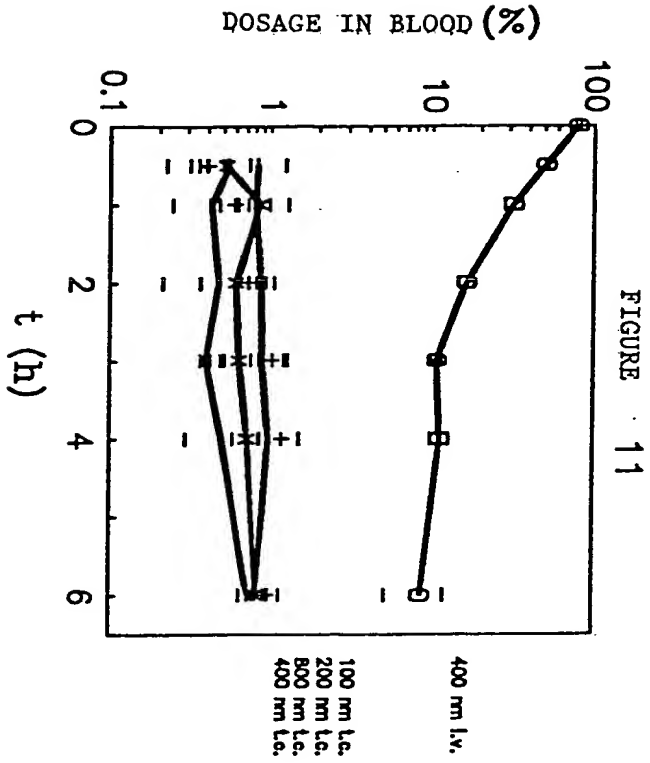

DOSAGE IN BLOOD (%)
0.1
1
10
100
0
2
4
6
t (h)
100 mm l.c.
200 mm l.c.
800 mm l.c.
400 mm l.c.
400 mm i.v.

FIGURE 11

FIGURE 12

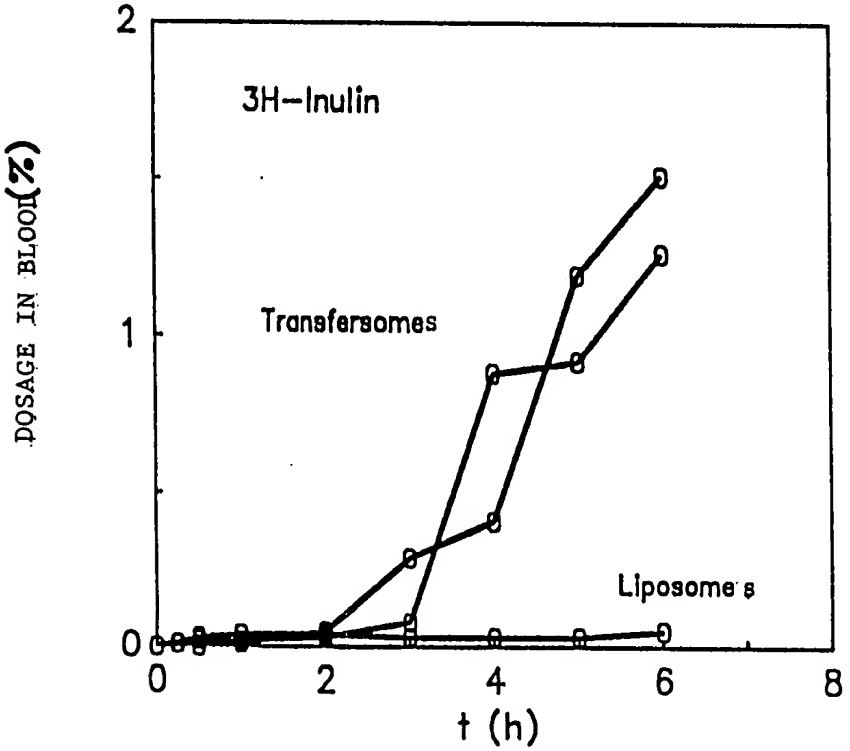

2
1
0
3H-Inulin
Transfersomes
Liposomes
DOSAGE IN BLOOD(%)
0
2
4
6
8
t (h)

FIGURE 13

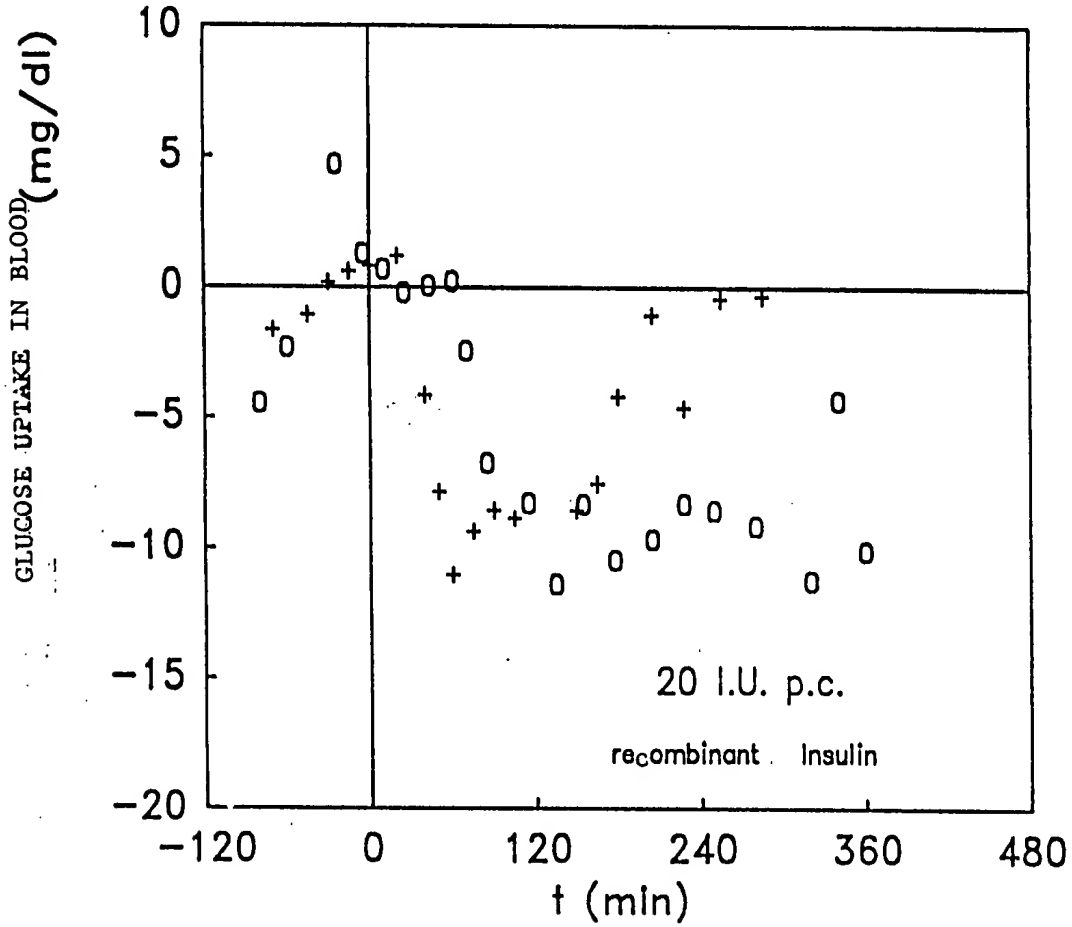

GLUCOSE UPTAKE IN BLOOD (mg/dl)
10
5
0
−5
−10
−15
−20
−120
0
120
240
360
480
t (min)
20 I.U. p.c.
recombinant Insulin

FIGURE 14

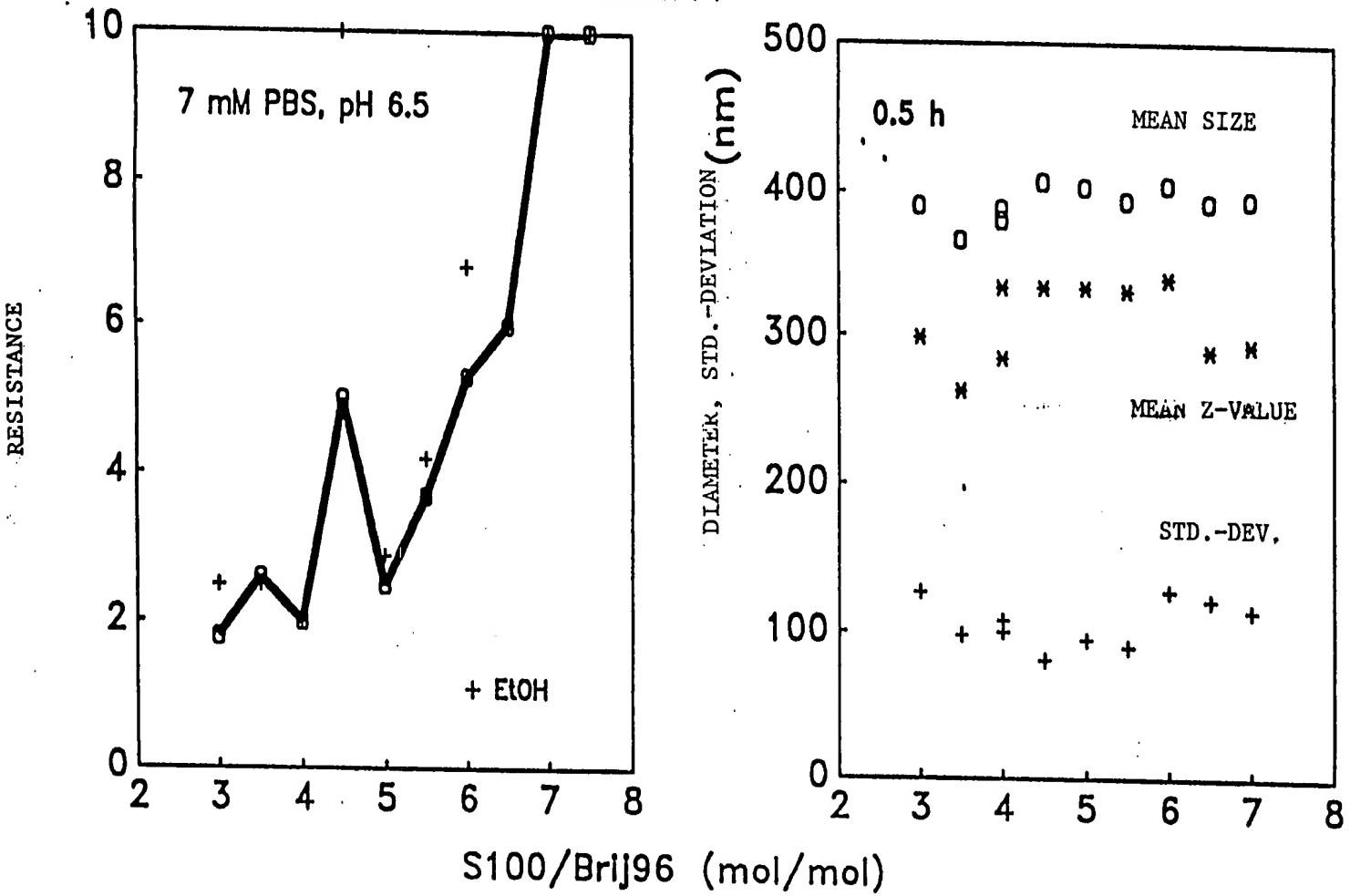

RESISTANCE
10
8
6
4
2
0
2
3
4
5
6
7
8
7 mM PBS, pH 6.5
+ EtOH
DIAMETER, STD.-DEVIATION (nm)
500
400
300
200
100
0
0.5 h
MEAN SIZE
MEAN Z-VALUE
STD.-DEV.
S100/Brij96 (mol/mol)

FIGURE 15

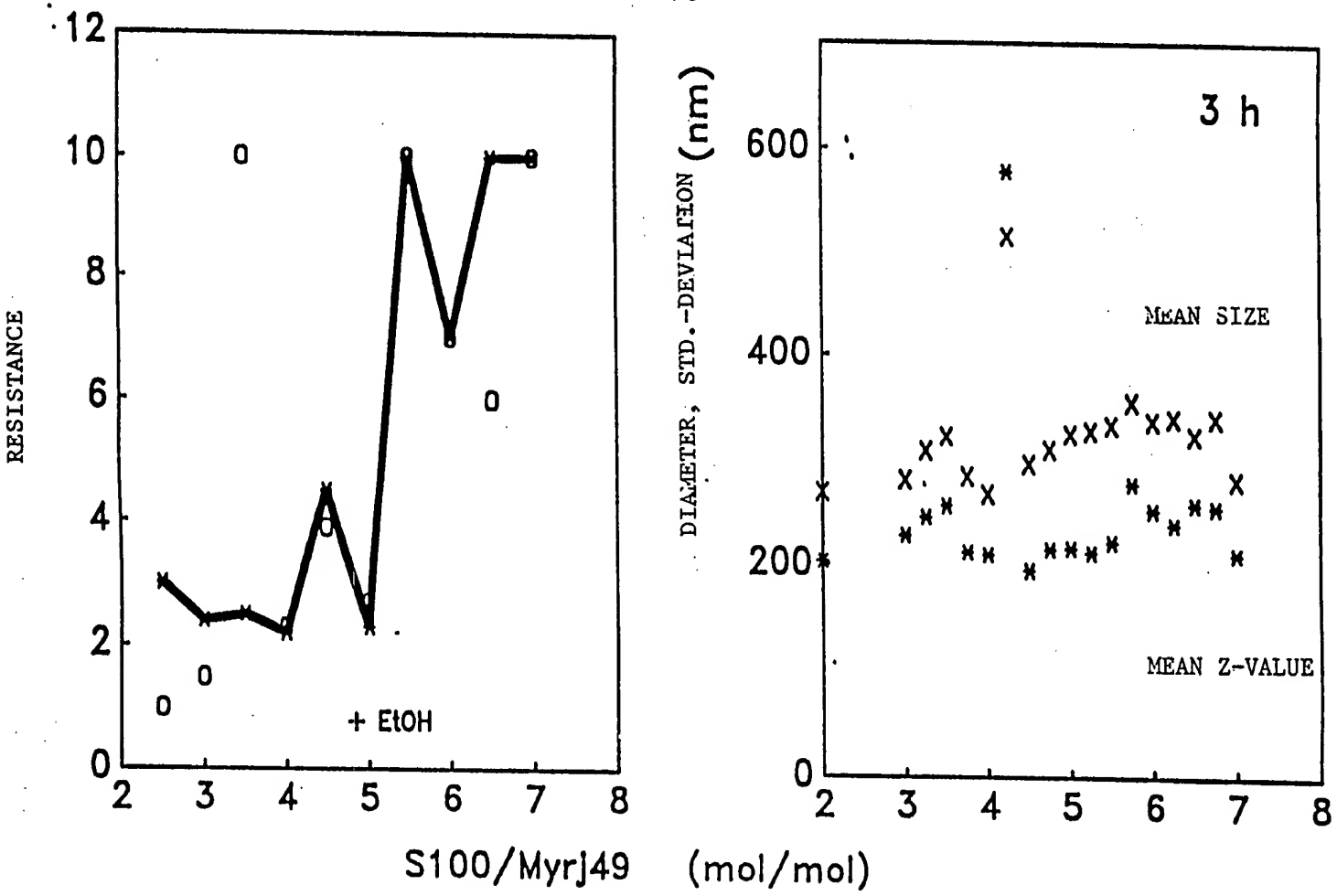

RESISTANCE
12
10
8
6
4
2
0
2
3
4
5
6
7
8
+ EtOH
S100/Myr]49 (mol/mol)
DIAMETER, STD.-DEVIATION (nm)
600
400
200
0
3 h
MEAN SIZE
MEAN Z-VALUE

FIGURE 16

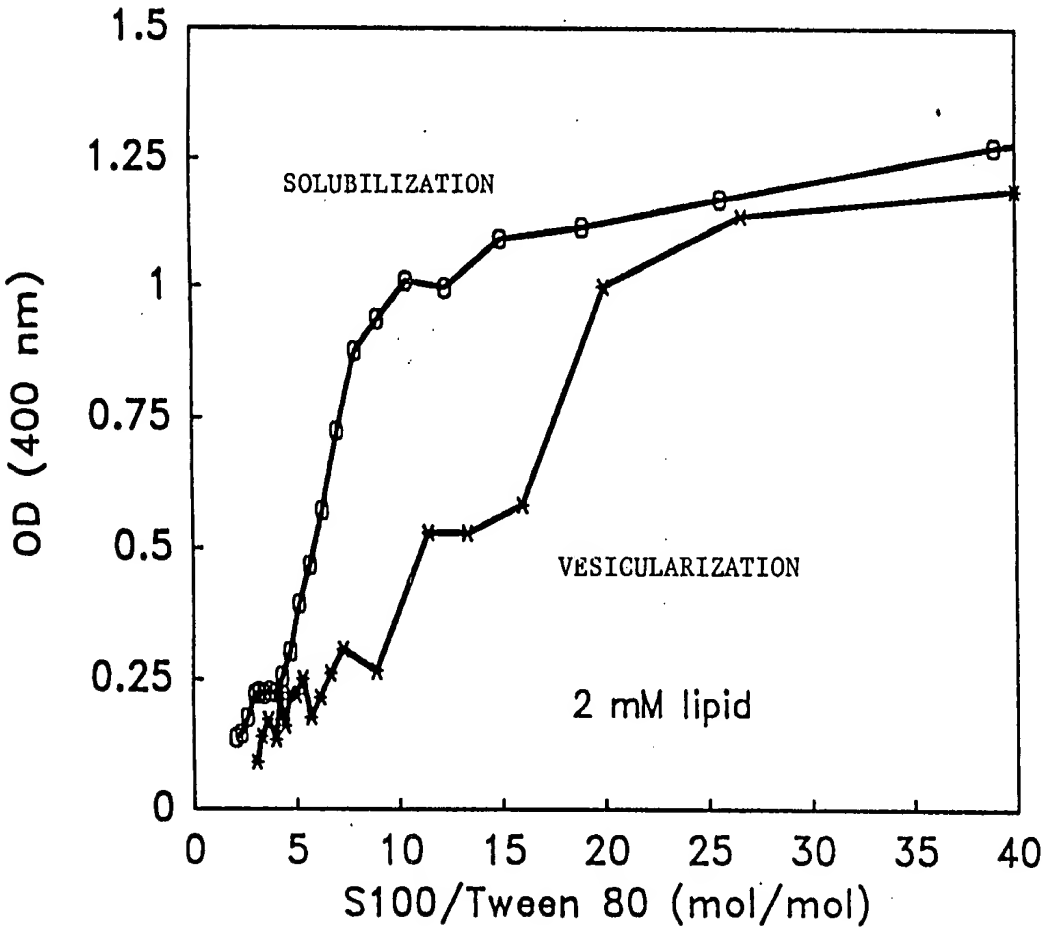
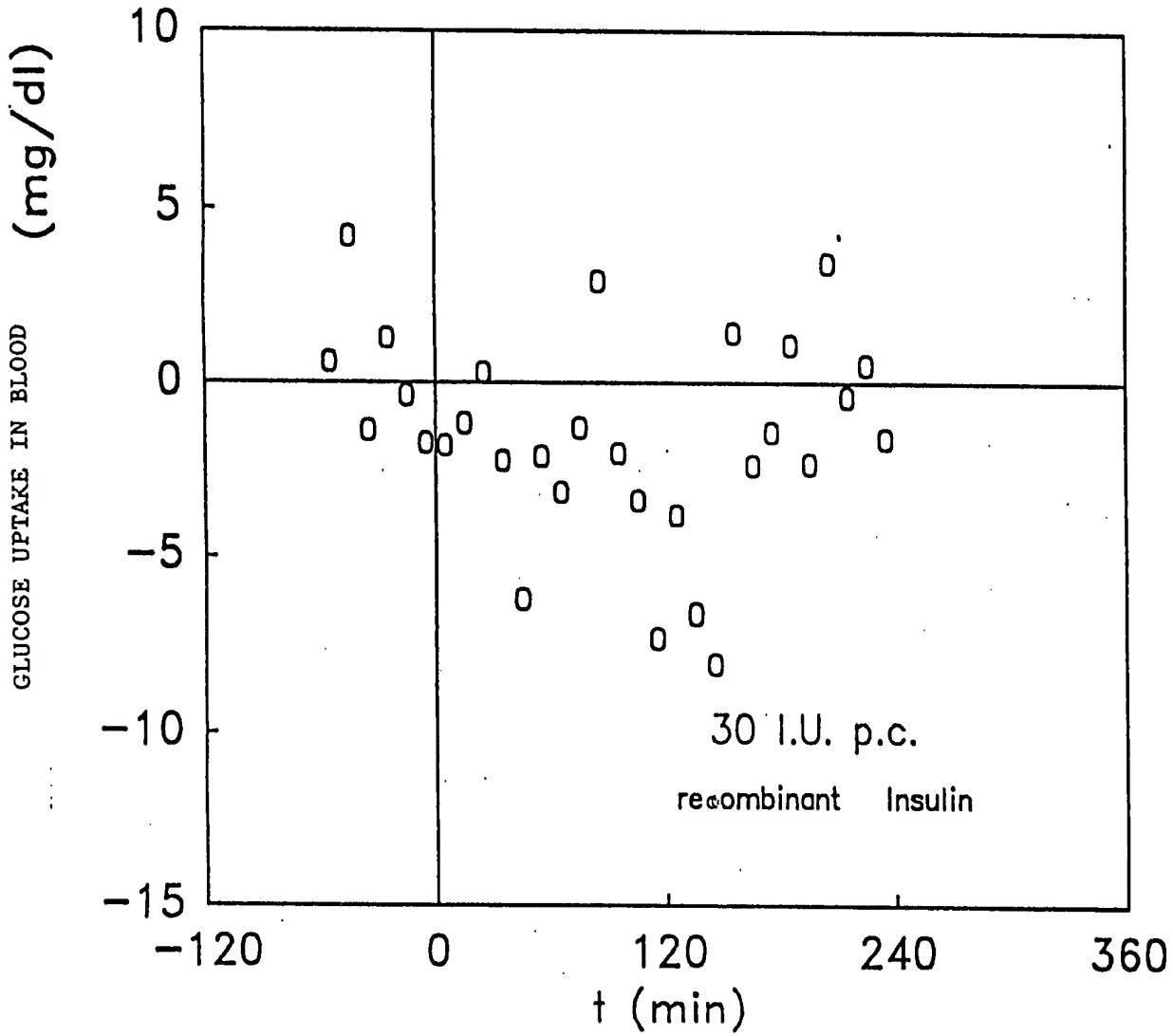

1.5
1.25
1
0.75
0.5
0.25
0
OD (400 nm)
SOLUBILIZATION
VESICULARIZATION
2 mM lipid
0
5
10
15
20
25
30
35
40
S100/Tween 80 (mol/mol)

FIGURE 17


GLUCOSE UPTAKE IN BLOOD (mg/dl)
10
5
0
−5
−10
−15
−120
0
120
240
360
t (min)
30 I.U. p.c.
recombinant Insulin

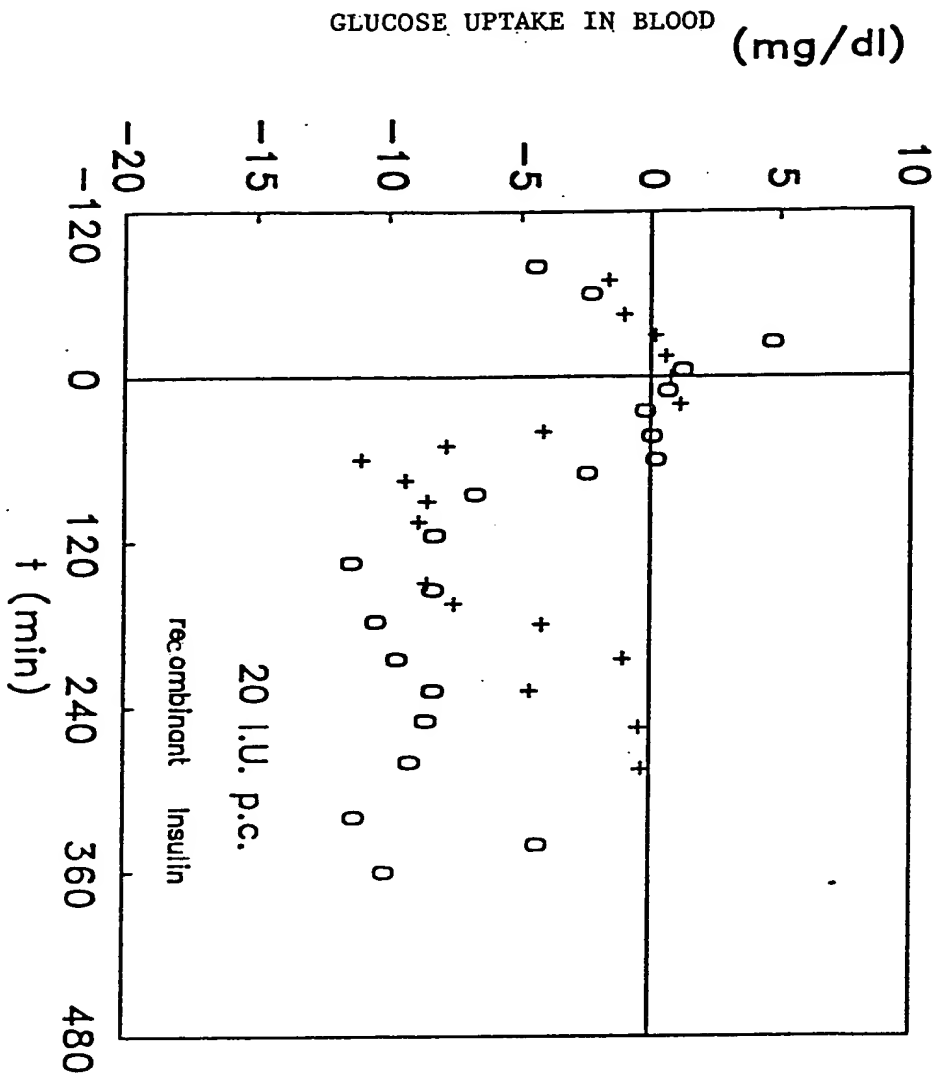

GLUCOSE UPTAKE IN BLOOD
(mg/dl)
10
5
0
−5
−10
−15
−20
−120
0
120
240
360
480
t (min)
20 I.U. p.c.
recombinant Insulin

FIGURE 18

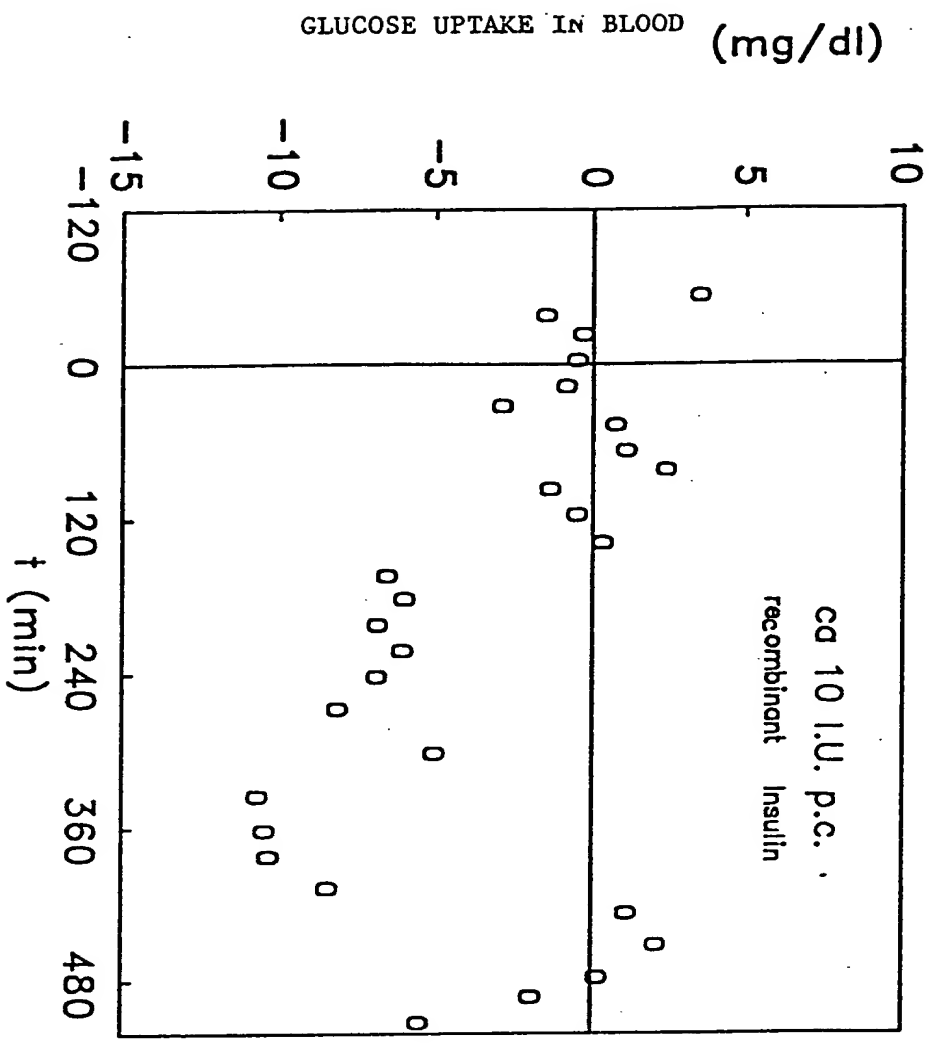

GLUCOSE UPTAKE IN BLOOD
(mg/dl)
10
5
0
-5
-10
-15
-120
0
120
240
360
480
t (min)
ca 10 I.U. p.c.
recombinant Insulin

FIGURE 19

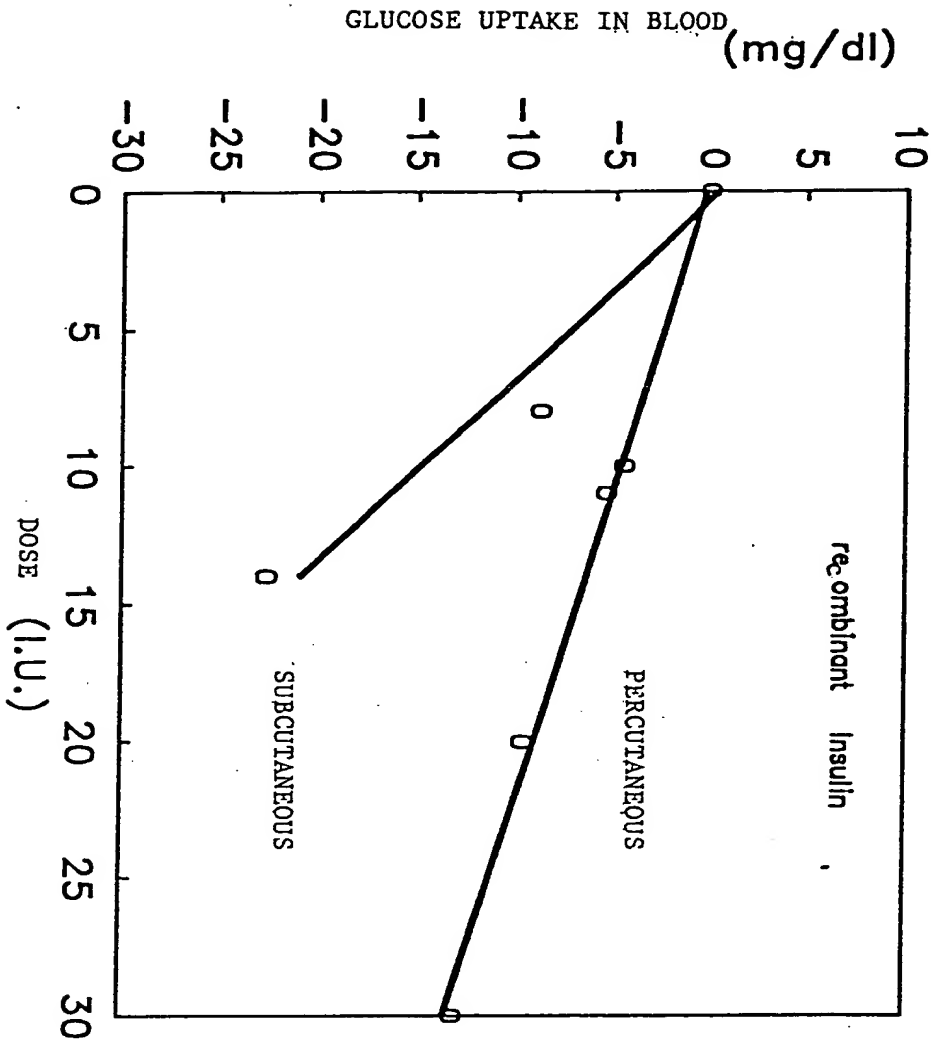
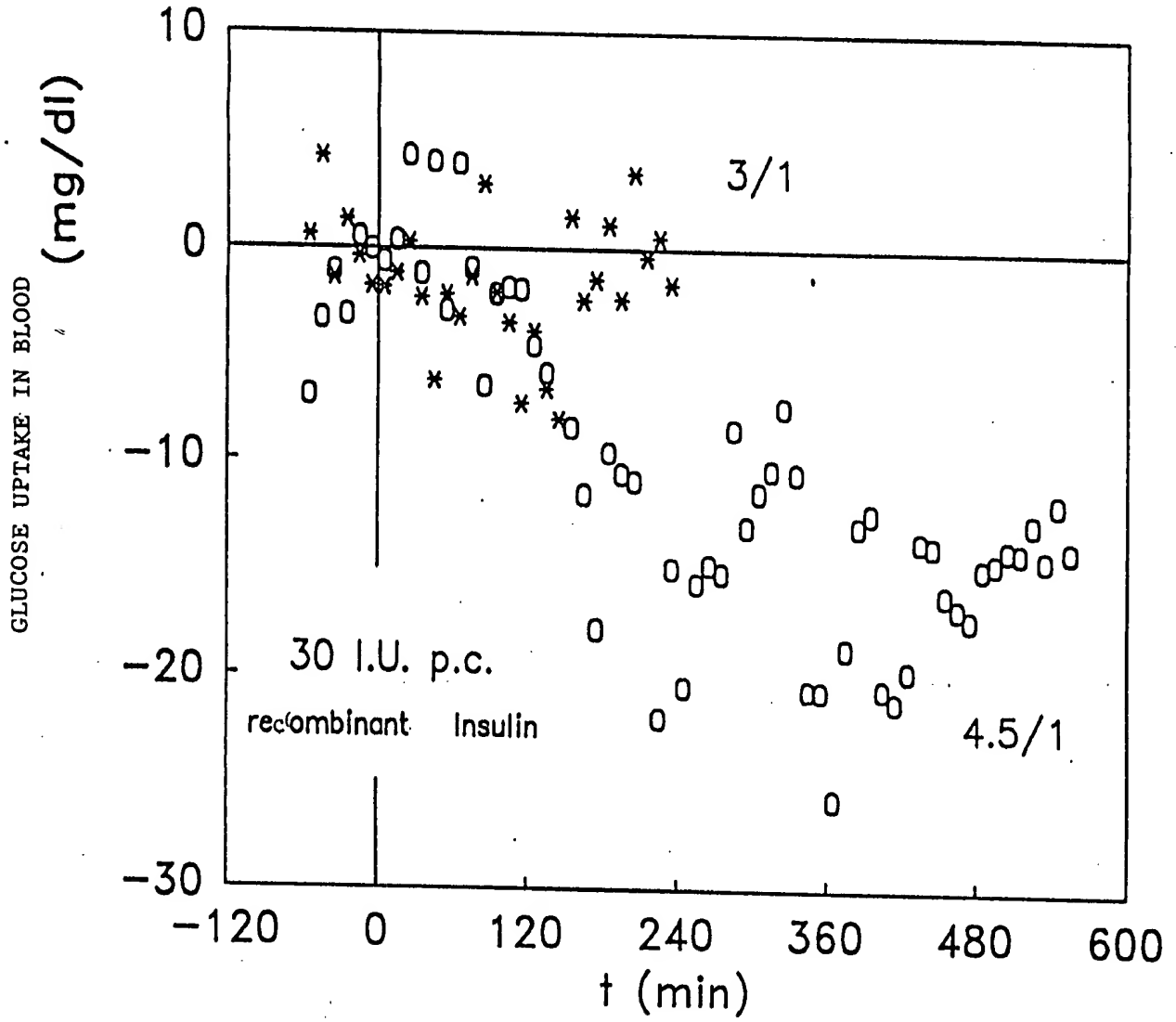

GLUCOSE UPTAKE IN BLOOD
(mg/dl)
10
5
0
-5
-10
-15
-20
-25
-30
0
5
10
15
20
25
30
DOSE (I.U.)
recombinant Insulin
PERCUTANEOUS
SUBCUTANEOUS

FIGURE 20

FIGURE 21


10
0
−10
−20
−30
(mg/dl)
GLUCOSE UPTAKE IN BLOOD
3/1
30 I.U. p.c.
recombinant Insulin
4.5/1
−120
0
120
240
360
480
600
t (min)